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Development and validation of the method of sublingual blood sampling in mice and other small rodents

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1 Summary / Zusammenfassung

The examination of blood is a key component in studies of laboratory animals. In rodents, retrobulbar venous plexus punction is the commonly used blood sampling method. This technique yields large volumes of blood but can cause severe tissue damage.
The aim of the study was to develop punction of the *V.sublingualis* as an alternative technique to be used for mice and other rodents. In rats this method is already established and is recommended in Switzerland.
In the validation part of the study, the retrobulbar and sublingual methods were compared using male CD1 mice.
The pilot study used 18 mice and the main study used 80 mice. 300µL of blood was collected from either the sublingual vein or the retrobulbar venous plexus.
No statistically significant differences in body weight, food or water consumption between the punctured groups were detected.
Differences between specific blood parameters were observed, depending on the technique used. Increased values for leukocytes, lymphocytes and monocytes were found in sublingual blood.
Retrobulbar blood yielded higher values for alanin aminotransferase, aspartate aminotransferase and glucose.
Mice punctured using the retrobulbar method showed more tissue destruction with a higher mean severity in histological slides.
The study's conclusion is that sublingual blood collection is a suitable method to use for mice and hamsters.
Because guinea pigs do not possess a sublingual vein, this technique is not suitable for this species.

Blutuntersuchungen nehmen in tierexperimentellen Studien eine zentrale Rolle ein. Werden grosse Volumina benötigt, stellt die Punktion des retrobulbären Veneplexus die gewöhnlich benutzte Technik bei Labornagern dar. Diese Methode birgt jedoch ein erhöhtes Risiko von Gewebeschäden in sich.
Ziel der vorliegenden Arbeit war, die Technik der Blutentnahme aus der *V.sublingualis* als Alternativmethode bei Mäusen und anderen Labornagern zu etablieren. In der Schweiz ist diese Technik bei Ratten inzwischen empfohlen.
Zur Validierung wurde die retrobulbäre mit der sublingualen Technik in männlichen CD1-Mäusen verglichen. 300µL Blut wurden jedem Tier entweder aus dem retrobulbärem Plexus oder der *V.sublingualis* entnommen.
Signifikante Unterschiede des Körpergewichts, Futter- und Wasserverbrauchs wurden nicht festgestellt.
Dagegen konnte ein technikabhängiger Unterschied einzelner Blutparameter festgestellt werden. Signifikant höhere Werte zeigte Blut der *V.sublingualis* bei Leukozyten, Lymphozyten und Monozyten. In Blut des retrobulbären Venenplexus wurden signifikant höhere Alanin Aminotransferase- und Aspartate Aminotransferase-Aktivitäten, bzw. Glucosegehalte gemessen.
Beim pathohistologischen Vergleich beider Techniken zeigte sich, dass retrobulbär punktierte Tiere mehr und gravierendere Schädigungen des punktierten Gewebes aufwiesen.
Die vorliegenden Ergebnisse zeigen, daβ die sublinguale Blutentnahme bei Maus und Hamster, nicht aber beim Meerschwein, geeignet ist.

2 Introduction

The examination of laboratory animals' blood and its parameters has elementary importance for many medical and toxicological studies and often is essential. Examples of frequently conducted studies using laboratory rodents include studies of drugs and drug metabolism in pharmacology research, antibody titers in immunology studies, and basic technical research. The choice of an ideal blood collection technique is a crucial aspect of an experimental schedule.
In line with the aim of the experiment, the selection of a blood sampling method depends on the purpose of the blood examination and on which type of blood (arterial, venous or a mixture of arterial and venous blood) is required. The blood volume required and the scheduled sampling frequency are also important, because this directly influences the choice of method.
If different techniques are available to achieve the aim of the study, the most harmless method has to be used, in order to minimize the stress and distress caused to the animals.
One of the most common blood sampling techniques for mice and to a lesser extent for guinea pigs and hamsters is the punction of the retrobulbar venous plexus. This method permits the collection of large volumes of blood but is associated with a great risk of tissue damage in the eye region. For this reason this technique may not be suitable for repeated sampling.
Many investigations have been published concerning the punction of the retrobulbar venous plexus. Most of the investigations of the retrobulbar technique, its risks and its influence on blood parameters, have been done with rats.
The anatomy of the eye and adnexa structures of mice, guinea pigs and hamsters is comparable to the rat's anatomy. Therefore references to studies using rats seem to be valid and necessary in this study.

In Switzerland, blood sampling from the retrobulbar venous plexus in rats is no longer recommended without special permission. If large volumes of blood are required, the punction of the sublingual vein, a method evaluated by ZELLER et al., is recommended (ZELLER et al., 1998).
This technique permits large-volume blood sampling that does not have a severe impact on the animals used. Today, retrobulbar punction in rats is a method that requires special justification, as better methods are available.

This study investigates the question of whether the method of retrobulbar punction can be refined by using a less severe form of the method in mice. The adaptability of the method to hamsters and guinea pigs is also investigated in this study.
In order to maintain clarity in this document, the three species are described separately in the introduction, the method chapter, the result chapter and the discussion.

2.1 Mouse

Laboratory mice are the most frequently used species in laboratory animal studies. As one of the most important animal models, the mouse is used in preclinical pharmacological and toxicological evaluations of drugs, especially anticancer drugs. Mice are also often used in immunological investigations. The preclinical assessment of drugs' toxicity requires extensive hematology, clinical chemistry and histopathology investigations.
Furthermore, mice are required for investigations of various diseases, including complex inflammatory responses. In these studies the evaluation of blood parameters, e.g. different peripheral blood cell counts, white blood cell differentials or parameters of cell adhesion molecules, often absolutely requires the availability of large volumes of blood.

The commonly used blood collection technique yielding large volumes is the punction of the retrobulbar venous plexus.
The retrobulbar technique is efficient but has the potential to cause severe tissue damage, especially if it is carried out by inexperienced researchers (HERCK et al., 1998). Therefore the development of an alternative method is useful.

Various techniques for the collection of minimal to large volumes of blood from mice have already been developed and have been published in the scientific literature.
National and international institutions and organizations have published special guidelines and recommendations regarding blood collection methods, including issues such as the volumes involved and the need for anesthesia. In some cases the recommendations differ widely, for example in their assessment of the necessity of anesthesia, the degree of severity, or whether a technique has to be terminal or not.
A great number of blood collection techniques have been developed for mice. These techniques can generally divided into groups according to the following aspects:
- techniques classified as terminal or non-terminal
- collectable volumes (small to moderate; moderate to large).
A detailed overview of the available blood sampling techniques for mice can be found in the Annex of this thesis.
This thesis investigates two methods: the punction of the retrobulbar venous plexus and the punction of the sublingual vein. Therefore this introduction basically focuses on these techniques.
The introduction consists of three main parts following the opening paragraphs. First, there is an overview of the literature on tissue damage resulting from retrobulbar venous plexus punction. Based on these results, the punction of the sublingual vein is described. The third main part of the introduction deals with investigations concerning the influence of blood sampling techniques on blood parameters.

Description of the retrobulbar technique and overview of tissue damage resulting from retrobulbar venous plexus punction

The technique of retrobulbar venous plexus blood collection was developed and described by PETTIT and is commonly accepted around the world as safe and effective (PETTIT, 1913). However, this procedure's potential for severe tissue damage has caused controversial discussion.
The retrobulbar venous plexus is also called the retroorbital plexus or periorbital sinus, even though these terms are not anatomically correct. The retrobulbar venous plexus fills the space between the orbita and the bulbus. Before puncturing, the mouse is anesthetized and placed on its left side if the right eye is to be punctured. With the thumb and forefinger the eyelid is pulled away by exerting a slight pressure on the bulbus, which leads to a slight protrusion of the bulbus.
The punction is carried out using a glass microcapillary or micropipette. The pipette or microcapillary is inserted into the medial or lateral canthus of the eye, passing the conjunctives with a quick rotating movement. Some authors believe the medial canthus is the ideal route because the retrobulbar venous plexus seems to be more easily reachable this way (METZKE, 1966). Blood appears in the lumen of the microcapillary/-pipette immediately if the plexus has been punctured successfully. To facilitate a stronger flow of blood, an adequate congestion of the head veins is necessary. This is achieved by compressing the skin of the neck. This compression should not be too tight, so as to avoid disabling the respiratory system of the animal during blood sampling.

A blood sample of 200µL and more can be collected with a micro blood collection tube (First report of the BVA / FRAME / RSPCA / UFAW Joint Working Group on Refinement, Appendix A, 1993).
If Pasteur pipettes are used, a maximum outer diameter of 1-2mm is recommended (HOFF, 2000).
In rare cases, secondary bleeding may appear after blood collection. Such persistent bleeding can occur especially if the congestion of the head veins is continued. Another possible complication is the occurrence of bleeding from the punction route parallel to the capillary if it is blocked by a grafting of the Harderian gland tissue (TILGNER and METZKE, 1964).

Although the punction of the retrobulbar venous plexus is generally described as reliable, this method has to be regarded as a questionable procedure.
Tissue damage resulting from retrobulbar bleeding has long been described in the scientific literature.

In 1979 McGEE and MARONPOT assessed local necrotic inflammations in the acini of the Harderian gland near the eye muscle tissue as a consequence of retrobulbar blood collection in rats (McGEE and MARONPOT, 1979). The authors show that the inflamed Harderian gland acini changed into fibrotic and atrophic tissue within four weeks. These inflammations had a focal character, which made it possible to differentiate them from the viral Sialodacryoadenitis, which always has a diffuse spread.

The histopathological alterations resulting from the retrobulbar blood sampling technique in rats were also investigated by MESSOW et al. (MESSOW et al., 1980).
The rats showed no macroscopic alterations, but microscopic changes with different grades of severity were found by means of a methodical examination of the eyes and the surrounding area in every animal.
The authors record hemorrhages and inflammations in general. The nature of the inflammations is described as purulent or purulent necrotizing. Acute inflammations of the Harderian gland and muscle tissue showed polymorphonuclear leukocytes in particular.
The alterations are divided into three groups:
- changes in the peripheral muscle and connective tissue, with the development of chronic granulation tissue (fibrosis)
- changes in the Harderian gland tissue, with the development of focal, partly high-grade granulation tissue (fibrosis) and, as a consequence, regeneration and metaplasia processes in glandular tissue as well as pronounced atrophy of single acini
- changes of the bulbus in the form of high-grade purulent processes (only occurring in certain cases).
Furthermore, the authors report that about 1-2% of the animals went blind as a result of multiple retrobulbar blood collections.

In a retrospective study, KRINKE et al. determined the influence of the age of an animal and the consequences of multiple retrobulbar blood collections on the retina, lens and Harderian gland of laboratory rats (KRINKE et al., 1988). This long-term study (with a duration of up to three years) notes the occurrence of retinal atrophy in rats after the withdrawal of large volumes of blood (up to 7mL/animal) from the retrobulbar venous plexus. The incidence of this alteration correlates with the volume of the collected blood.

An investigation by HERCK et al. dealt with the temporal development of retrobulbar lesions induced by single or double retrobulbar punction in Wistar rats at intervals of 14 days (HERCK et al., 1992).

The authors noticed hemorrhages in the punction area and, depending on the technique, in the periost. These effects were caused by a single retrobulbar blood collection.
On day 4 after the blood collection, infiltrates of monocytes, fibroblastocytes and histiocytes were apparent in histological slides. These signs of inflammation could not be found on day 28 *post punctionem*.
Furthermore, the study detects the influence of the nature of the instruments and the technique used on tissue lesions. The authors come to the conclusion that the use of a broken hematokrit capillary causes less tissue damage than a Pasteur's pipette. In addition, reaching the osseous orbita with the capillary during blood collection seems to be more painful, because this method causes additional hemorrhages and probably inflammations in the *orbita* even though it guarantees an adequate depth of puncture.

In another investigation using rats, HERCK et al. proved that the technician's degree of experience influences the incidence of lesions, the grade of severity and the prognosis for the animal (HERCK et al., 1998).
If carried out by an unskilled technician, retrobulbar blood sampling has a greater potential to result in complications than other methods (U.S. Department of Health and Human Services; National Institute of Health, 2005).
Because of the aforementioned investigations by HERCK et al. among others, the Animal Care and Ethics Committee of the University of Newcastle ruled that retrobulbar venous plexus punction is a non-recommendable technique if the blood collection is not carried out as a terminal procedure (Animal Care and Ethics Committee of the University of Newcastle, 1999).

In Switzerland, the technique of retrobulbar blood collection in mice is considered permissible if a blood volume larger than 0.1mL is required and if the procedure is carried out in an anesthetized animal by a skilled technician only. If repeated collections are required, the punctured eye has to be given a recovery period of at least 14 days before the same eye is punctured again. Two weeks are regarded as a satisfactory period for the regeneration of damaged tissue (Bundesamt für Veterinärwesen, BVET – Richtlinie 3.02).

Punction of the sublingual vein as an alternative method for rats – an overview of the literature

In 1998 ZELLER et al. published a new method of blood collection in rats (ZELLER et al., 1998).
The authors refined an old blood collection technique that involved the incision of the sublingual vein with eye scissors in rats (ANGELOW et al., 1984).
Since the study of ZELLER et al. was published, the method of blood collection from the sublingual vein has been considered a suitable method for rats and is recommended in the Swiss guidelines (Bundesamt für Veterinärwesen, BVET – Richtlinie 3.02).

This method permits the collection of blood volumes comparable to those that can be collected by means of the retrobulbar technique, and is carried out with rats under anesthesia using a 23-gauge hypodermic needle that punctures the sublingual vein. Even multiple collections during 24 hours are feasible in rats. Water and food consumption as well as body weight gain are affected only by the required anesthesia. This result was also reported by NAHAS et al. (NAHAS et al., 2000).

MAHL et al. compare the effects of repeated blood samplings using the traditional retrobulbar blood sampling technique and the sublingual method in a pharmacokinetic study (MAHL et

al., 2000). In this study, rats punctured by the retrobulbar method showed an initial decrease of leukocytes (especially of lymphocytes) and higher activities of creatin kinase and aspartate aminotransferase compared to the parameters of rats whose blood was collected by the sublingual method. The authors argue that the technique of blood collection from the retrobulbar venous plexus caused more severe tissue damage than did the punction of the sublingual vein.

So far, if large blood volumes are required for a study, only blood collection from the retrobulbar venous plexus has been available as a non-terminal procedure in addition to terminal techniques in mice. This method may be associated with potentially severe tissue damage and consequent distress of the punctured mouse. In addition, the procedure requires an experienced technician and may be regarded as a burden for the personnel.
For these reasons, it makes sense to search for an alternative method in mice that yields comparable blood volumes of comparable quality without causing severe physical damage.
As mentioned before, the punction of the sublingual vein is accepted as an alternative method for rats and is recommended as the preferable method in Switzerland (Bundesamt für Veterinärwesen, BVET – Richtlinie 3.02).
On the basis of this background, the punction of the sublingual vein is investigated in this study to find out whether this method is suitable as an alternative method in mice as well.

Investigations concerning the influence of blood sampling techniques on blood parameters

Numerous studies have demonstrated the influence of the chosen blood collection method on hematology and clinical chemistry parameters. The hematology and clinical chemistry parameters differ considerably if procedures are compared to one another (e.g. MAHL et al., 2000).
The investigation of DAMERON et al. is linked to this context (DAMERON et al., 1992). The authors do not recommend the use of retrobulbar venous plexus blood sampling if plasma coagulation times are measured in rats. The study compares the technique of retrobulbar venous plexus blood collection with the technique of collecting blood from the *V.cava posterior*. The results show significantly prolonged prothrombin and partial thromboplastin coagulation times for blood samples collected from the retrobulbar venous plexus when compared with samples collected from the posterior *V.cava*.
The authors assume that tissue damage caused by retrobulbar punction probably activates plasma coagulation factors. As an alternative hypothesis, the authors presume that the plasma coagulation factors are consumed during the blood sampling.

In a study of HERCK et al. single blood collections from the retrobulbar venous plexus, the saphenous vein and the tail vein in rats were compared (HERCK et al., 2001). The study demonstrates the differences between selected parameters which are influenced by the respective technique.
Blood taken from the saphenous vein showed a higher count of erythrocytes, hemoglobin and hematocrit than blood taken using the two other methods. The retrobulbar blood collection yielded the lowest erythrocyte, hemoglobin and hematokrit counts.
Blood collected from the retrobulbar venous plexus showed a significant higher pCO_2 and sodium as well as a significant lower pH and potassium compared to blood taken from the saphenous vein and the tail vein. The authors assume that the reason for these parameter differences is that less damage is done to the erythrocytes by the retrobulbar technique and that this technique has less influence on intracellular and extracellular barriers. Furthermore,

none of the tested blood collection methods had a statistically significant influence on selected parameters of the behavior of the animals (locomotion, grooming and inactivity).
In a comparative study SCHNELL et al. determined that there were significant differences between the clinical pathology parameters of blood taken from mice using either the heart, the *V.cava caudalis* or the retrobulbar venous plexus as the source of blood samples (SCHNELL et al., 2002). The authors concluded that different techniques of blood collection yielded significant different findings regarding clinical pathology parameters.
In this study, blood collected from the retrobulbar venous plexus showed an increase in erythrocyte, hemoglobin and hematokrit counts compared to blood collected by other techniques. This differs from the results of the investigation of HERCK et al., 2001) which is mentioned above.
There are also differences between the types of enzyme activity. Blood taken from the retrobulbar plexus yielded higher activities of aldolase, serum transaminase and alkaline phosphatase compared to blood collected using other blood sampling methods.
Furthermore, a marked increase of aspartate aminotransferase was seen in blood from the retrobulbar venous plexus. The authors explain the increase of enzyme activity as a consequence of retrobulbar tissue lesions or damage to erythrocytes.

DOEING et al. investigated the influence of different blood collection techniques and the influence of sex on peripheral total leukocytes in mice (DOEING et al., 2003). In this study, blood from male and female mice was taken from the heart, the tail vein, the foot vein and the saphenous vein. The study demonstrated no variation between the leukocyte counts for all the techniques except for the heart punction. Leukocyte counts in cardiac blood showed significantly lower levels.
No blood parameter differences between male and female mice was observed except for the heart punction. Blood taken from female mice showed a significantly lower percentage of neutrophile granulocytes than blood taken from male mice. The authors suppose that murine sex hormones have a regulatory effect on circulation leukocytes. Therefore DOEING et al. recommend that researchers maintain constancy in the design of studies of blood sampling techniques, especially with regard to the gender of the animals, if leukocyte counts are the topic of the investigation.

Concerning the variability of leukocyte counts as a result of the technique used, the study of NEMZEK et al. shows different results compared to the above cited study (NEMZEK et al., 2001). This study compares parameters of blood obtained from the heart, the retrobulbar venous plexus and the tail tip of anesthetized female BALB/c mice. Compared to other sources, the results show a significant increase of leukocyte counts in tail vein blood. Compared to the tail vein blood, samples obtained from the heart show the lowest white blood cell counts. Retrobulbar blood yields moderate white blood cell counts compared to heart or tail blood.
The authors conclude that the sampling site influences the total and differential counts of blood cells, red blood cells and platelets in anesthetized mice. Therefore they recommend that researchers standardize sampling sites if these parameters are being investigated in research studies.

Several studies compare the blood parameters of blood samples collected from the sublingual vein and the retrobulbar venous plexus of rats. The results of a parameter comparison yield important information regarding the two techniques applied to mice.
In 1984 ANGELOW et al. published a comparative study that shows minimal differences between the hematology and clinical chemistry parameters of blood obtained from male rats using the sublingual (incision by scissors) and the retrobulbar blood sampling method

respectively (ANGELOW et al., 1984). The differences are evaluated as non-significant and are described as a slightly increased hematokrit and total protein level, as well as a slightly decreased triglyceride level in sublingual blood samples compared to the retrobulbar samples. More apparent results are described in the investigation of MAHL et al. comparing the sublingual (using the refined method of punction) with the retrobulbar technique (MAHL et al., 2000). Repeated blood samplings from anesthetized Hanover Wistar rats were investigated in this kinetic study. The authors describe clear differences between the relevant blood parameters, depending on the technique used. Compared to the sublingual blood samples, the blood taken from the retrobulbar venous plexus yielded lower lymphocyte counts as well as increased neutrophil counts, creating kinase or aspartate aminotransferase activity respectively. The authors conclude that retrobulbar blood sampling results in more severe tissue damage than the sublingual method.

A summary table of the investigations mentioned concerning the influence of the blood sampling technique on blood parameters can be found in the Annex.
As shown by these studies, the blood sampling technique used may have a significant influence on several blood parameters. Therefore, an analysis of this aspect is included in this thesis.

2.2 Guinea pig

The guinea pig (*Cavia porcellus*) is considered an important laboratory animal, and is commonly used in studies of, e.g., immunology, otology, asthma and infectious diseases. Studies concerning the production of polyclonal antibodies and the collection of complements for in vitro essays frequently require the guinea pig as an animal model (TERRIL and CLEMONS, 1998).

Depending on the volume of blood required, various blood collection techniques are also available for guinea pigs. Some of these techniques are not satisfactory, e.g. with regard to the collectable volume or the simplicity of the procedure. More information about this issue can be found in the Annex.

A common blood sampling technique that yields moderate to large volumes of blood is retrobulbar punction in anesthetized guinea pigs. Comparable to the procedure in mice, a heparin hematokrit capillary is inserted into the medial canthus of the eye and pushed forward while it is being rotated. Blood can be sampled with micro blood collection tubes. Subsequent to the blood collection, gentle pressure should applied to the eye region to prevent persistent bleeding (TERRIL and CLEMONS, 1998).
This method is classified as feasible if volumes larger than 150µL are required. The procedure has to be carried out exclusively by experienced technicians in order to minimize the risk of tissue damage. According to Swiss regulations, a second punction of the same eye is permitted after at least 14 days of recovery (Bundesamt für Veterinärwesen, BVET – Richtlinie 3.02).

The punction of the retrobulbar venous plexus in general requires technical experience. The technique can cause potentially severe damage or subsequent complications.
Therefore, the adaptation of the sublingual venepuncture that was described as a suitable technique for rats by ZELLER et al. (ZELLER et al., 1998) seems to be an alternative method for guinea pigs. The adaptation of this technique for this species is defined as another aim of this thesis.

2.3 Hamster

The Syrian golden hamster (*Mesocricetus auratus*) ranks among the species of small laboratory rodents that are used in a wide variety of experimental setups. The examination of blood is an eminent factor in these studies.
Numerous blood collection techniques are described in the scientific literature. These techniques differ in their complexity and the degree to which they are successful or satisfactory.
Terminal and non-terminal techniques are available for use with hamsters.
Further information about blood collection techniques in hamsters is given in the Annex of this thesis.

One of the most common blood sampling techniques for hamsters is the punction of the retrobulbar venous plexus. This method requires general anesthesia and is suitable for the collection of amounts of blood larger than 300µL from a hamster weighing 100g (PANSKY et al., 1961). As described above, this technique has the potential to damage tissue in the eye region in hamsters as well as in other rodents. For this reason, Swiss law generally permits this technique to be carried out only by skilled persons on an anesthetized hamster (Bundesamt für Veterinärwesen, BVET – Richtlinie 3.02).
This method is known to be harmful to punctured rodents, and although it is satisfactory in terms of its collectable volume, it has to be regarded very critically.

The available methods for blood collection in hamsters are not convincing. They are either classified as terminal procedures or they permit the collection of only small volumes of blood. In addition, some of the techniques are connected with a great risk of damage to tissues or organs.
None of the blood sampling methods is ideal for hamsters in general, especially if large volumes or repeated collections in non-terminal schedules are planned.
For these reasons, an investigation of the adaptability of the sublingual blood sampling method to hamsters was scheduled.

3 Aims of the thesis

3.1 Mouse

The central aim of this study is the development and validation of the sublingual blood sampling technique for mice. First, the technique is established, and then the retrobulbar technique is compared to the sublingual method of blood collection. The purpose is to compare the retrobulbar technique to the developed sublingual blood collection method. Particular attention is paid to the influences of both techniques on in-life development and their potential to damage tissue.
Of particular interest is the detection of significant differences between the blood parameters (hematology and clinical chemistry parameters) of the blood collected using each of the two techniques.

3.2 Guinea pig

The aim of this part of the study is to adapt the technique of sampling blood from the *V.sublingualis* so that it can be used for guinea pigs. The goal is the technical development of this technique and its suitability for this species.

3.3 Hamster

The evaluation of the feasibility of the sublingual vein punction technique for hamsters is the purpose of this segment of the study. As in the guinea pig study, only technical development is planned.

4 Animals, materials and methods: Mouse

This study was conducted under animal experimentation license number 5093 according to Swiss animal welfare legislation.

4.1 Animals

Male Crl: CD1 (CR) mice (breeder: Charles River Wiga GmbH, Sulzfeld, Germany) were used. The animals were eight weeks old when delivered. The mean body weight was 35.7g on day 1 of the study (ranging between 32.0 and 39.3g). The choice of age and weight was in accordance with standard common practice at Novartis Toxicology, Switzerland.
After delivery, a microchip (DATA MARS SA, 6930 Bedano – Lugano, Switzerland) was implanted subcutaneously in every animal under isoflurane anesthesia. After that, 13 days (pilot study) or 16 days (main study) were given to the animals for acclimatization. The difference between the two acclimatization periods was due to organizational reasons.

4.2 Animal husbandry

The male mice were individually caged in type III macrolone boxes with autoclaved wood chip bedding and a mousehouse® (Tecniplast, Germany). Autoclaved paper tissues were put on the cover of every single cage every day for environmental enrichment. The distribution to every single cage and to experimental groups was carried out using a random list, as is usually done in Novartis toxicology studies. The random list was created according to internal standard operation procedures, that base upon Swiss regulation of good laboratory praxis.
The cages were placed in a room with a controlled temperature between 20–24°C, a relative humidity of 40-70%, a light management of a 12h light period and a 12h dark period (max.100 lux light intensity in the cages between 6 a.m. and 6 p.m.), and quiet background radio music from 6 a.m. until 6 p.m.
A traditional rodent diet was fed (Provimi KLIBA SA, Kaiseraugst, Switzerland - mouse/rat husbandry "GLP" NAFAG 890) *ad libitum*. Fresh water was available from 300mL macrolone bottles.
The cage with bedding was replaced once a week. The water bottles were changed three times a week. Food was monitored every day and changed once a week (new food was given from the same batch).

4.3 Experimental design

A pilot study using 18 male mice and a main study using 80 male mice were carried out.

4.3.1 Pilot study

The main purpose of the pilot study was to evaluate the schedule and the procedure (especially the use of the TOX DATA computer system, a program designed to capture data in toxicological studies; TOX DATA version 7.5; SeAG Software Engineering AG) and, additionally, to observe the clinical signs following blood samplings.
As shown in figure 1, 18 mice divided into two groups of 9 mice each were scheduled for the pilot study. Blood was taken from the mice in the first group using the sublingual blood collection technique. Blood was taken from the mice in the second group using the traditional technique of retrobulbar venous plexus punction. 300µL of blood was collected from every mouse under isoflurane anesthesia.

Each group was divided into 3 subgroups of 3 animals. The schedule included one subgroup for hematology and two subgroups for clinical chemistry examination. The maximum collectable amount of blood per animal (300µL) just sufficed for the hematological examination. An additional clinical chemistry examination could not be scheduled because of this small amount. For this reason, a subgroup was scheduled for hematology only. In addition, two subgroups for different clinical chemistry examinations were planned.
The food and water consumption, the development of body weight, and the clinical signs (e.g. swelling, haematoma or persistent bleeding) were measured in both groups. To monitor the animals' behavior, paper tissue was provided on the cover of every cage (unreferenced control of tissue paper use).
No control group (= without treatment) was defined.

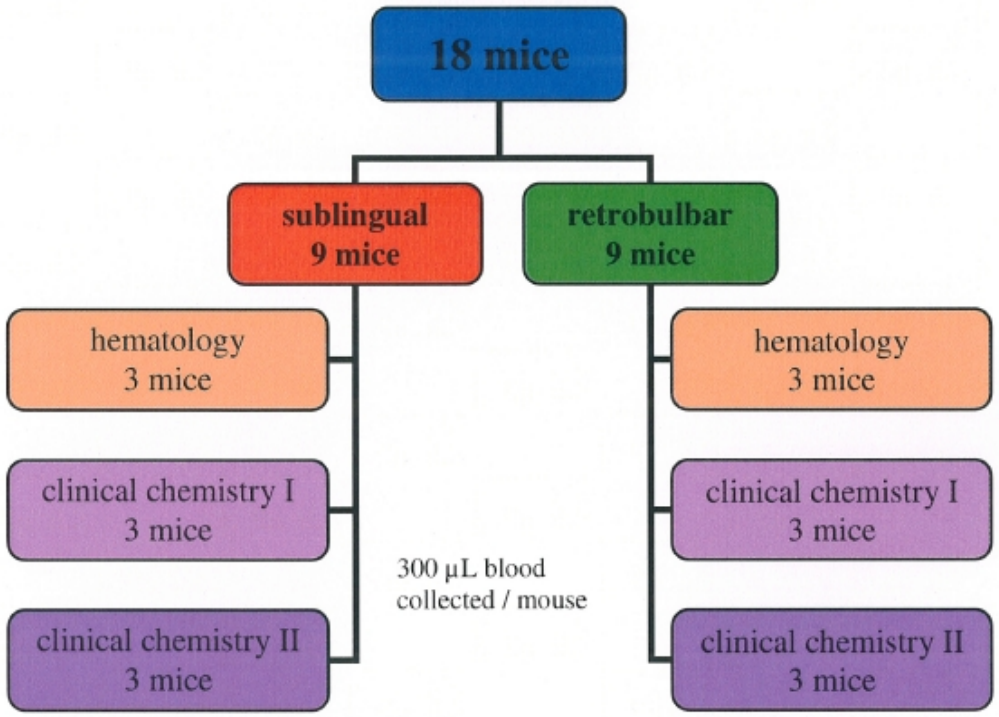


Figure 1: Illustration of the group distribution of the 18 mice in the pilot study

The eyes of the 9 mice punctured by the retrobulbar method were examined by an experienced technician using indirect ophthalmoscopy (retrobulbar group). The tongue's lower sides of the 9 mice punctured by sublingual method were examined under general anesthesia (sublingual group). In order to prevent the results regarding alimentary development in the main study from being influenced by any stress or anesthesia, only the tongue region of the mice in the pilot study was examined.
Histological slides were made of the tongue or eye region, including the orbita.

4.3.2 Main study

The aim of the main study was to determine the development of body weight as well as food and water consumption. Additionally the comparison of differences in blood parameters resulting from the two different techniques (blood collection from the sublingual vein versus from the retrobulbar venous plexus) was purposed.
80 mice were divided into 4 groups (shown in figure 2):

30 male mice were used for the sublingual punction technique and an additional 30 male mice were used for the retrobulbar blood sampling technique. By analogy with the schedule of the pilot study, both groups were divided into 3 subgroups of 10 animals. The blood of the animals in the first subgroup was analyzed for its hematology parameters. The other subgroups were used for two different clinical chemistry parameter ranges. As in the pilot study, 300µL of blood was collected from each mouse.
Unlike the pilot study schedule, two control groups were defined in the schedule of the main study. A control group of 10 male mice was housed without exposure to anesthesia or blood samplings (control I).
Another control group consisting of 10 male mice was exposed to anesthesia only (control II). These animals were not punctured.

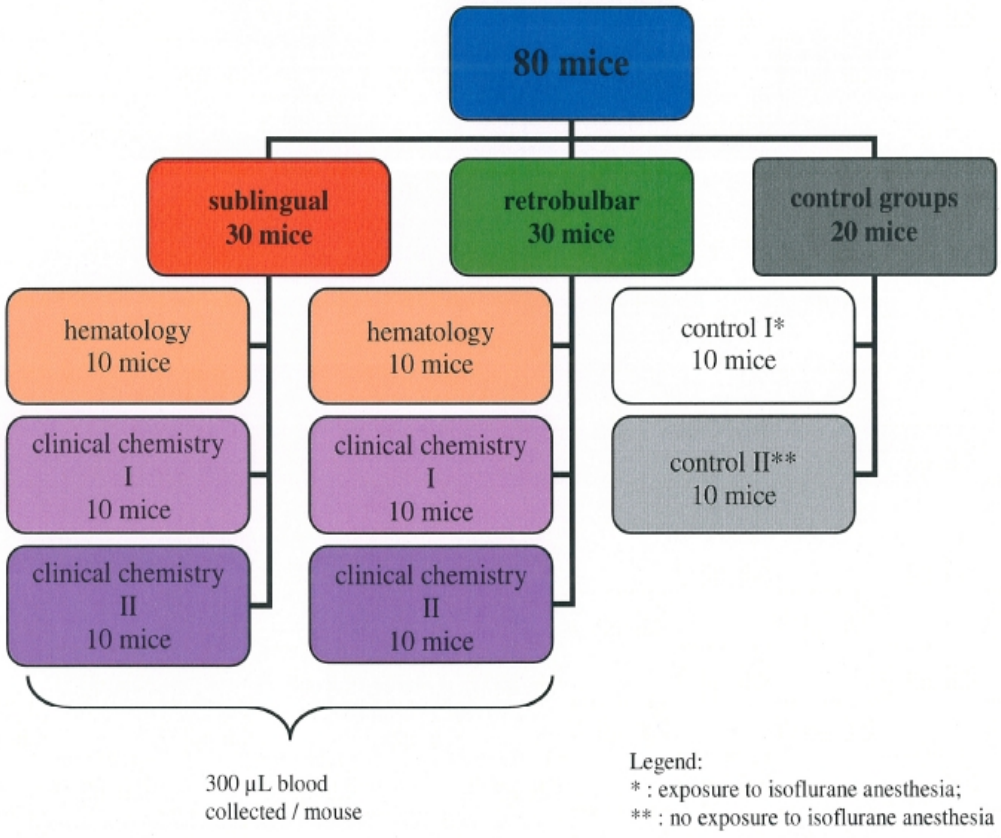


Figure 2: Illustration of the group distribution of the 80 mice in the main study

4.4 Anesthesia

Mice were brought into an inhalation chamber (size: 25 x 17 x 20cm) and exposed to short-term inhalation anesthesia with 3% isoflurane anesthesia (Forene™, Abbott Laboratories S.A., Switzerland) followed by the two different blood sampling techniques and the sublingual examination. An isoflurane vaporizer (Vet.Anest 2004 Vaporizer Fluotec 3) and a medical oxygen carrier (flow rate 4L/min) were used. The animal's period of unconsciousness lasted 40–50 sec. This time span permitted blood collection using one of the two techniques.

4.5 Technique of sublingual blood sampling

The anesthetized animal was taken by its tail and kept in a headfirst position during 2-3 seconds. After that the animal's neck skin was grasped in order to assure a partial congestion of the jugular and lingual veins. The mouse was brought into a supine position.
A second person extended the tongue by picking it up between the thumb and a cotton bud (under a bright light). This is shown in Figure 3.

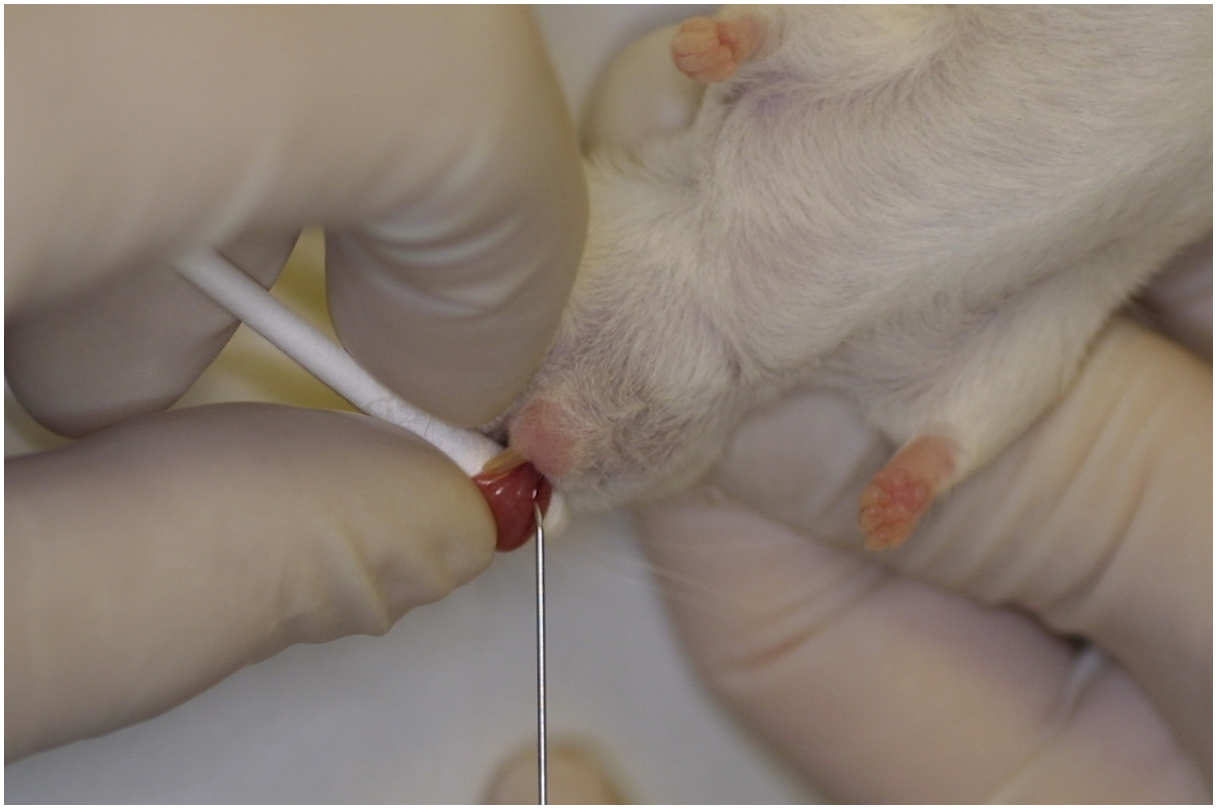


Figure 3: Handling of a mouse that is about to be punctured using the sublingual technique

The thick caudal part of the left *V.sublingualis* was punctured with a 24-gauge (24 G x 1"; 0.55 x 0.25mm) hypodermic needle.
Blood was collected into a micro blood collection tube holding the mouse in a horizontal position above the tube.
A volume of 300µL was collected from every mouse.
To stop the congestion, the grasp of the animal's neck skin was released. The mouth of the mouse was cleaned with a dry cotton bud to remove any remaining blood.
The collection of blood from every mouse generally lasted 20–30 seconds, in some cases only 12-15 seconds.

4.6 Technique of retrobulbar blood sampling

The retrobulbar technique was carried out by an experienced technician to minimize the amount of damage to the eye tissue.
The anesthetized mouse was placed on its left side. With the thumb and forefinger, the lids of the right eye were pulled away by applying a slight pressure to the bulbus, leading to a protrusion of the bulbus.

The punction was carried out using a 20µL non-heparin glass micro hematokrit capillary. The capillary was inserted at a 45-degree angle into the medial cantus of the eye, passing the conjunctives with a quick rotating movement.
Next, the skin of the neck was grasped between the thumb and forefinger to create an adequate congestion of the head veins and a stronger flow of blood. Blood dropped immediately from the capillary end, and 300µL of blood was collected into a micro collection tube. The release of the neck skin and the removal of the capillary stopped the bleeding immediately.
Only the right eyes were punctured. The non-punctured left eyes were used as control tissue.

4.7 Investigations

4.7.1 Monitoring of individual animals

The TOX DATA computer system (TOX DATA version 7.5; SeAG Software Engineering AG) was used to keep daily records of body weight, food and water consumption, and clinical signs of behavioral changes or illness (e.g. swellings in the eye region, persistent bleeding in the eye or mouth region) of every single mouse. The mice were weighed with a calibrated balance. The same system was used to calculate the daily food and water consumption and to document clinical signs.
The daily individual monitoring of clinical signs, body weight, and food and water consumption started on day 1 and was finished on day 13, when the necropsy was done. These measurements were carried out between 7:30 a.m. and 10 a.m. every day (weekends included). For further details see Table 1.

4.7.2 Investigation of lingual damage

The macroscopic investigation of damage to the sublingual region of the sublingually punctured mice was carried out under 3% isoflurane anesthesia (Forene™, Abbott Laboratories S.A., Switzerland). The examination was done three days before blood collection, two hours after blood collection, and one day and three days after blood collection respectively.
Only sublingually punctured mice from the pilot study were examined in this way, in order to avoid influencing the daily individual monitoring of the mice in the main study. The anesthetized mice were put on their backs. Using a cotton bud and the thumb of the same hand, the tongue was rolled out of the animal's mouth and fixed between the thumb and forefinger. The lower surface of the tongue was inspected for haematoma, swellings, discontinuity of the vein or other signs of damage (without grading system).
Notwithstanding the study schedule, the investigation of the tongue was not done a day after blood collection because of an error. Therefore this investigation was carried out using a representative group (using 9 mice from the former control group of the main study after this part of the study was finished).

4.7.3 Hematology and clinical chemistry

Becton Dickinson Microtainer ™ tubes with EDTA anticoagulant (Becton, Dickinson and Company, USA) were used for hematology.
Micro blood collection tubes for serum sampling with a separating gel (Sarstedt AG, 51588 Nümbrecht, Germany) were used for clinical chemistry.

For hematology findings an ADVIA 120 (Bayer Diagnostics, Munich, Germany) was used. This apparatus delivers an automated full program of hematological parameters that demands a minimal amount of blood totaling 300µL per animal and examination. Total white blood

cells, differential white blood cells, red blood cells, hemoglobin, hematokrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and platelet counts were defined as the parameter range.
Blood smears were prepared for an optical microscopic check of the appearance of aggregations of platelets.
The clinical chemistry parameters alanine aminotransferase, albumin, alkaline phosphatase, aspartate aminotransferase, glucose, triglyceride, urea, calcium, chloride, potassium, sodium, and total protein were determined with a Synchron CX5 Beckmann Coulter.

4.7.4 Ophthalmoscopic examination

The eye and any potential damage induced by the retrobulbar technique were examined by a technician who had experience with this type of investigation.
Only mice punctured using the retrobulbar method were examined. In addition to the punctured right eye, the non-punctured left eye was also examined as a control organ.
An indirect ophthalmoscope (Keeler All Pupil) with 25 and 90 diopters was employed two hours after the retrobulbar blood collection without using mydriatic eye drops, in order to avoid further irritation to the eye. The indirect ophthalmoscopy took place one day and three days after the retrobulbar blood sampling – first without and then with the use of mydriatic eye drops (Mydriaticum Dispersa, pharmaceutically active substance Tropicamidum, Novartis Pharma AG, Switzerland).
Additionally, a basic examination of the health status of the eyes was done 4 days (pilot study) or 3 days (main study) before the blood sampling was carried out. All examinations included the outer parts of the eye as well as the fundus of the eye and the intraocular structures (anterior and posterior chamber of the eye, iris, lens and retina). No severity grades of observed damages were defined. The ophthalmoscopy required restraint by a second person but no anesthesia.
The ophthalmoscopy examinations were carried out in the same time frame that was used for the examinations of sublingual damage.

4.7.5 Histopathology

The necropsy was done 6 days after blood collection (day 13 of the study). All mice used in the pilot study and only the mice punctured using the sublingual or retrobulbar method in the main study were macroscopically and microscopically examined.
Experienced pathologists performed the histopathology examination. One pathologist carried out the macroscopic examination and the preparation of the histological slides and another pathologist appraised the microscopic slides and assessed all results.
An overdose of carbon dioxide was used for euthanasia.
The tongues of the sublingually punctured mice and the orbital regions of both eyes of the animals punctured using the retrobulbar method were fixated in 10% formalin, embedded in Paraplast™ and stained with Hematoxilin–Eosin.
The punction area at the base of the tongue was cut transversally in an approximately 3-4mm block that was then split into two segments. These segments were embedded separately in Paraplast™ and transversal serial sections were prepared.
A modified transversal serial section along the optic nerves was carried out in the animals of the retrobulbar group.

4.7.6 Overview of activities in the pilot study and the main study

Table 1 gives an overview of the time schedule and the daily activities during the pilot study and the main study. The two parts of the study started on different days of the week. This resulted in differences in the acclimatization period (12 and 15 days respectively) and different dates for the basic examination of the eye.

Table 1: Time schedule and activities of the pilot study and the main study

Pilot study	Day	Activity		Main study	Day	Activity
Delivery	-12	Implantation of microchip		**Delivery**	-15	Implantation of microchip
Pretest	1 - 2	BW, FC, WC, tissue test, symptoms		**Pretest**	1 - 3	BW, FC, WC, tissue test, symptoms
	3	Basic examination of eyes/retrobulbar group BW, FC, WC, tissue test, symptoms			4	Basic examination of eyes/retrobulbar group BW, FC, WC, tissue test, symptoms
	4 – 6	BW, FC, WC, tissue test, symptoms			5 - 6	BW, FC, WC, tissue test, symptoms
Blood collection	7	300µL/mouse Examination of eyes/retrobulbar group after 2h BW, FC, WC, tissue test, symptoms		**Blood collection**	7	300µL/mouse (no punction of control mice!) Examination of eyes/retrobulbar group after 2h BW, FC, WC, tissue test, symptoms
	8	Examination of eyes/retrobulbar group Examination of tongue/sublingual group BW, FC, WC, tissue test, symptoms			8	Examination of eyes/retrobulbar group BW, FC, WC, tissue test, symptoms
	9	BW, FC, WC, tissue test, symptoms			9	BW, FC, WC, tissue test, symptoms
	10	Examination of eyes/retrobulbar group Examination of tongue/sublingual group BW, FC, WC, tissue test, symptoms			10	Examination of eyes/retrobulbar group BW, FC, WC, tissue test, symptoms
	11 – 12	BW, FC, WC, tissue test, symptoms			11 - 12	BW, FC, WC, tissue test, symptoms
Necropsy	13	BW, FC, WC, tissue test, symptoms Necropsy		**Necropsy**	13	BW, FC, WC, tissue test, symptoms Necropsy

Legend: BW: body weight, FC: food consumption, WC: water consumption
tissue test: monitoring to see whether tissue is brought through cage cover and a nest is built by mouse

4.8 Statistical analysis

For statistical analysis of body weight, food and water consumption the Dunnett test was used. The Dunnett test is based on pooled variance significant at the 5% or 1% level respectively. Both control groups and the sublingual group were compared to the retrobulbar group (reference group).
The hematology and clinical chemistry data were analyzed with the Student t-test significant at the 1% level. In the box the plots of the arithmetic mean and the percentiles (5% and 95%) are illustrated.
The data were presented using SigmaPlot, version 8.0.

5 Animals, materials and methods: Guinea pig

This study was conducted under animal experimentation license number 5095 according to Swiss animal welfare legislation.
In this study segment, two albino shorthair guinea pigs [Crl:(Ha)] of another Novartis exploratory study scheduled for necropsy were used for experimental sublingual punction.
The animals were anesthetized with isoflurane (3.5% with 4L/min oxygen flow) in an inhalation chamber (size: 25 x 17 x 20cm).
The unconscious guinea pig was placed in a supine position, and the tip of its tongue was pulled out of its mouth using a cotton bud and fixed between cotton bud and thumb. A blind testing punction of the supposed position of the sublingual vein (compared to the vein position of other rodents) was done.
A histological investigation of the tongues of the two guinea pigs was scheduled to find out the position of the sublingual vein, assuming that this vein exists in this species at all.
The tongues were separated and fixated in 10% formalin, embedded in Paraplast™ and stained with Hematoxilin–Eosin. One tongue was used for the preparation of serial transversal slides and the other tongue was used for longitudinal serial slides.

6 Animals, materials and methods: Hamster

This study was conducted under animal experimentation license number 5093 according to Swiss animal welfare legislation.

6.1 Animals

For this part of the study, 5 female and 5 male Syrian golden hamsters (HsdHan:AURA) aged 5 to 8 weeks at delivery were used. A total of 11 days were given for acclimatization before the blood collection was done.

6.2 Animal husbandry

The hamsters were housed in groups (separated by gender) in type IV macrolone cages with autoclaved wood chip bedding, a mousehouse®, an autoclaved board roll and straw.
The housing and feeding conditions corresponded to those of the mice (see above).

6.3 Anesthesia

The hamsters were exposed to short-term inhalation anesthesia with 4 percent isoflurane anesthesia (Forene™, Abbott Laboratories S.A., Switzerland) in an inhalation chamber (size: 25 x 17 x 20cm). An isoflurane vaporizer (Vet.Anest 2004 Vaporizer Fluotec 3) and medical oxygen as the carrier (flow rate 4L/min) were used. The animals were unconscious for 40–50 sec. This time span allowed the researchers to carry out the sublingual blood collection technique.

6.4 Technique of sublingual blood sampling

The sublingual blood collection was carried out by two persons. One person held the unconscious hamster, grasping the neck skin and presenting its ventral surface to the second person. The second person carried out the punction of the sublingual vein.
A cotton bud was put into the hamster's mouth and the tongue was pulled out of the mouth with rolling movements the cotton bud around its axis.
The tongue was fixed between the cotton bud and the thumb of one hand. Using a 24-gauge (24 G x 1"; 0.55 x 0.25mm) hypodermic needle, the left sublingual vein was punctured. The hamster was brought into a ventral position above a micro blood collection tube, into which the blood was collected.
A total of 5 hamsters (3 male and 2 female) were used for the hematology examination (500µL of blood was collected from each hamster). In addition, 5 other hamsters (2 male and 3 female) were used for the clinical chemistry examination (750µL of blood was sampled from each animal). The same type of micro collection tube was used both here and for the mouse study (see above). Releasing the tight grasp of the animal's neck skin interrupted the bleeding.
Figure 4 illustrates the sublingual vein of a hamster that is about to be punctured.

<u>Figure 4:</u> Left *V.sublingualis* of a hamster prepared for punction

Remark: A magnified section of the lower surface of the tongue is inserted into the left side of this photograph.

7 Results

7.1 Mouse

7.1.1 Individual animal monitoring

The results of the main study are presented exclusively.

Table 2 summarizes the data obtained in the main study: body weight, food and water consumption in both punctured groups as well as in both control groups. Only the data for days 5, 7, 8, 9 and 11 are shown.

Table 2: Individual animal monitoring (mean/standard deviation) on selected days

Group	Parameter	Day 4-5	Day 6-7 [a]	Day 7-8	Day 8-9	Day 10-11
Retrobulbar	BW / (g)	35.7 / 1.6	35.7 / 1.7	35.1 / 1.7	35.1 / 1.8	35.3 / 1.9
	FC / (g)	5.5 / 0.7	6.8 / 1.0	5.1 / 1.0	5.1 / 0.7	5.4 / 0.6
	WC / (mL)	7.1 / 1.3	6.4 / 1.1	6.9 / 1.4	6.2 / 1.1	6.5 / 1.1
Sublingual	BW / (g)	35.6 / 1.4	35.7 / 1.3	35.3 / 1.4	35.4 / 1.4	35.5 / 1.3
	FC / (g)	5.5 / 0.5	6.8 / 1.1	5.1 / 1.0	5.3 / 0.5	5.5 / 0.6
	WC / (mL)	7.2 / 1.2	6.5 / 0.9	6.7 / 1.2	6.4 / 1.2	6.9 / 1.2
Control I [b]	BW / (g)	35.6 / 1.3	35.9 / 1.1	36.2 / 1.5	36.2 / 1.4	36.2 / 1.5
	FC / (g)	5.7 / 0.9	7.0 / 1.2	5.9 / 1.1	5.9** / 0.8	5.7 / 0.7
	WC / (mL)	7.7 / 1.3	7.0 / 0.7	8.1 / 1.6	7.8** / 0.9	7.8** / 1.1
Control II [c]	BW / (g)	35.4 / 1.6	35.8 / 1.7	35.8 / 1.6	36.1 / 1.4	36.1 / 1.6
	FC / (g)	5.7 / 0.7	7.0 / 1.0	5.8 / 0.6	5.9** / 0.8	5.8 / 0.6
	WC / (mL)	7.3 / 0.7	6.4 / 0.8	7.3 / 1.1	7.6 **/ 1.0	7.5 / 1.0

Legend: BW = body weight; FC = food consumption; WC = water consumption
a = blood collection was done on day 7
b = no exposure to isoflurane anesthesia
c = exposure to isoflurane anesthesia
** = Dunnett test based on pooled variance significant at the 1% level

Compared to the retrobulbar group, no significant difference in body weight or food and water consumption was detected in the sublingual group. By contrast, both control groups show a statistically significant difference in food and water consumption on day 9. Mice of both control groups ate and drank significantly more ($p<0,01$) than did the mice in the retrobulbar group (reference group). Additionally, control group I (not exposed to anesthesia) showed a higher water consumption on day 11 compared to the retrobulbar group.
The distribution of mice into groups was randomized. No significant difference between the values for body weight or food and water consumption for the different groups was seen, either between days 4-5, days 6-7 nor days 7-8. Against this background, the data for days 4-5 and 6-7 were chosen to present the values for body weight and food and water consumption before the blood collection was done.

Control of tissue paper use
Every animal used the tissue paper that was provided on the top of the cage as nesting material. No differences were seen between the behavior of the 80 mice regarding group membership and treatment of the other animals.

Body weight

The results for the retrobulbar group, the sublingual group and both control groups of the main study are illustrated in Figure 5 (mean ± standard deviation are shown).

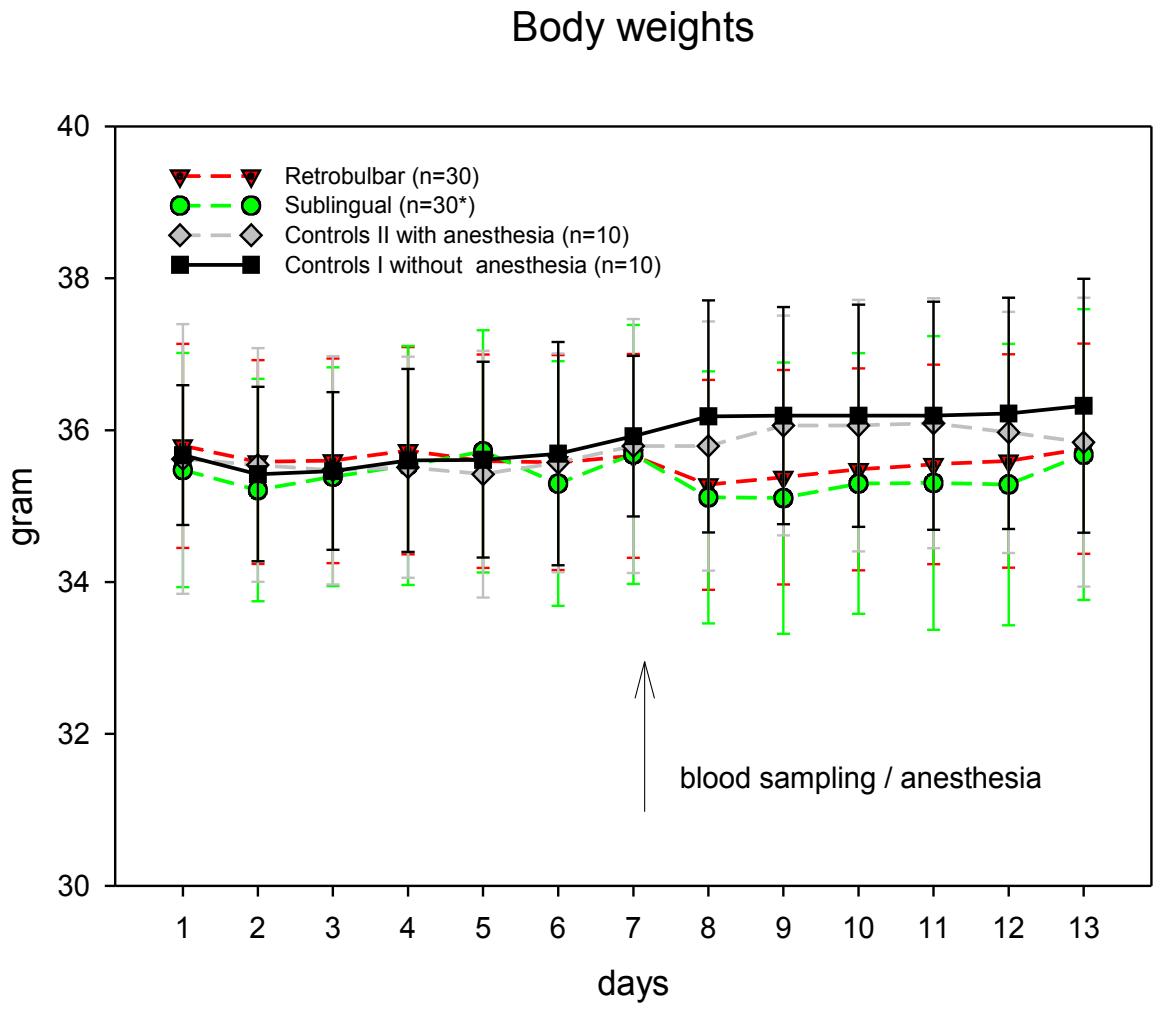


Figure 5: Body weight development of retrobulbar, sublingual and control groups

Legend: * = On day 7 one animal died during the blood collection, which reduced the number of animals in the sublingual group to n=29

After blood collection (on day 7 of the study), body weight decreased slightly in punctured animals compared to the day before sampling. The body weight of both control groups increased daily, as is normal for untreated mice.

Food consumption

The development of food consumption during the main study is illustrated in Figure 6 (mean ± standard deviation are shown).

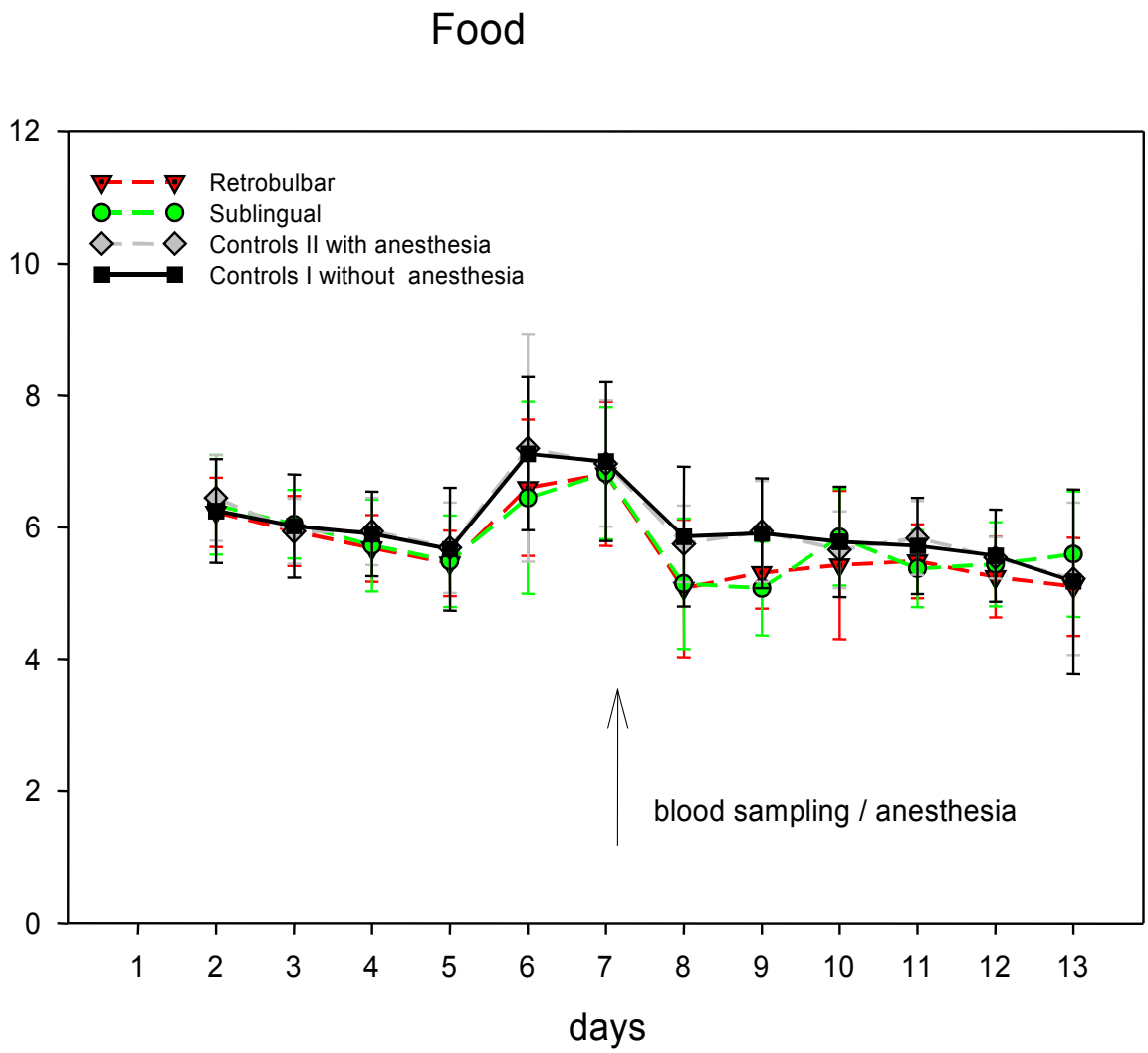


Figure 6: Food consumption of retrobulbar, sublingual and control groups

Legend: * = On day 7 one animal died during the blood collection, which reduced the number of animals in the sublingual group to n=29

The curves of both control groups and both punctured groups run in parallel.
From days 2 to 5 the mean intake in all groups was about 6 grams per day. From day 5 to 6 and from day 6 to 7 all groups showed an increase of about 1 gram per 24 hours.
All groups therefore decreased their food consumption from day 7 to 8, i.e. during the first 24 hours following the blood collection. The decrease was less pronounced in the control groups than in the two punctured groups.

Water consumption

The graphs of all groups are illustrated in Figure 7.

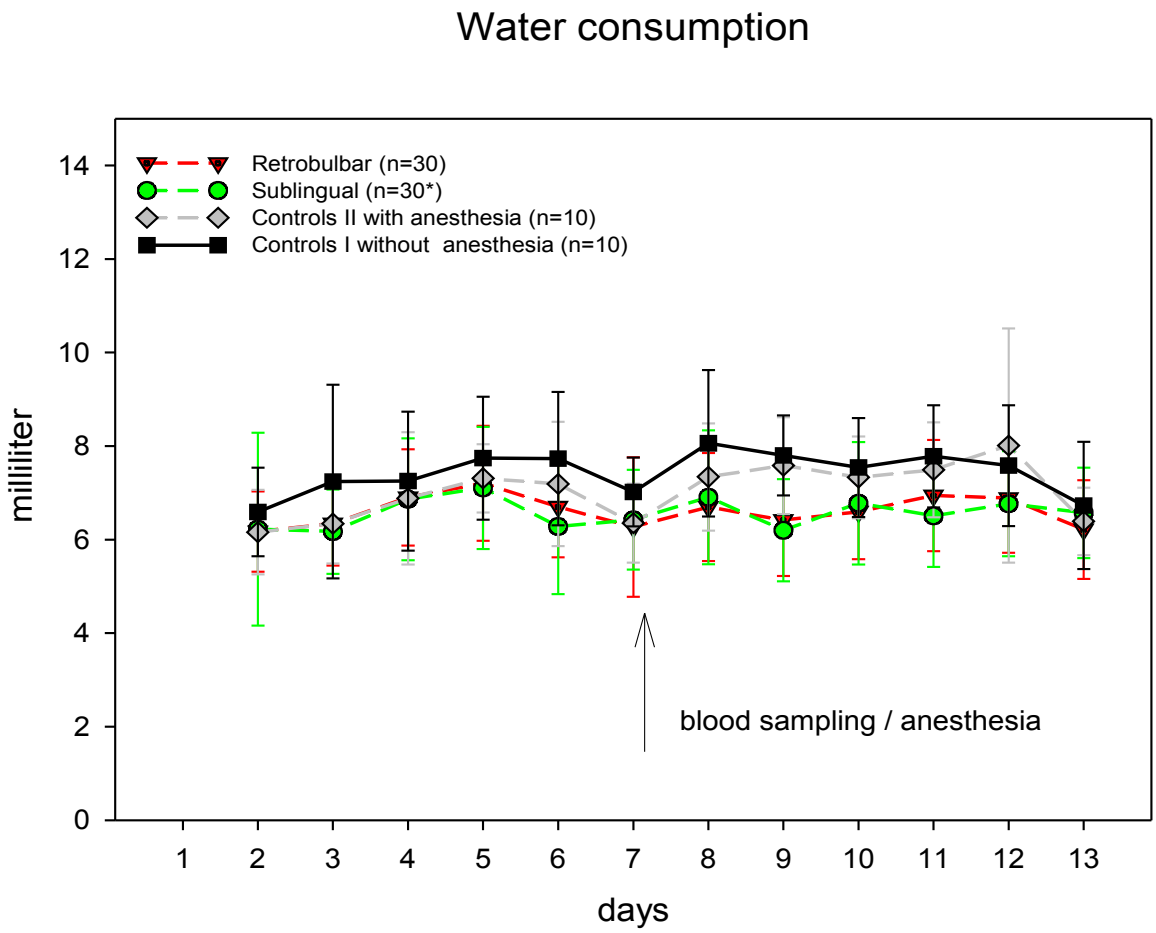


Figure 7: Water consumption of retrobulbar, sublingual and control groups

Legend: * = On day 7 one animal died during the blood collection, which reduced the number of animals in the sublingual group to n=29

The graphs of the control groups as well as the retrobulbar group and the sublingual group run in parallel. There was no statistically significant difference between the groups.

The water / food consumption ratio documents normal values and is shown in Table 3.

Table 3: Ratio of water consumption to food consumption for selected days

	Day 4-5	Day 6-7	Day 7-8	Day 8-9	Day 10-11
Control I	1.35	1	1.37	1.32	1.37
Control II	1.28	1.09	1.26	1.29	1.29
Sublingual	1.31	0.96	1.31	1.21	1.3
Retrobulbar	1.3	0.94	1.35	1.22	1.2

7.1.2 Blood collection technique

7.1.2.1 Blood collection from the sublingual vein

The method of sublingual venipuncture was developed for use in mice. The anesthetized mouse was grasped by its neck skin and brought into a supine position. The tongue was extended and the thick caudal part of the left *V.sublingualis* was punctured with a 24-gauge hypodermic needle. Blood was collected in a micro tube while the mouse was held in a horizontal position. This method made it possible to draw the required blood sample of 300µL from each animal without problems in approximately 20–30 seconds per animal.
Although the sublingual vein is visible along its entire length, only the punction of the thicker caudal part, an area of 2–3mm, yielded a satisfactory blood flow. If the punction was done closer to the apex of the tongue, only small amounts could be collected and there was also a risk of hemorrhage and subsequent swelling. The ideal location for punction of the sublingual vein in mice is illustrated in Figure 8.

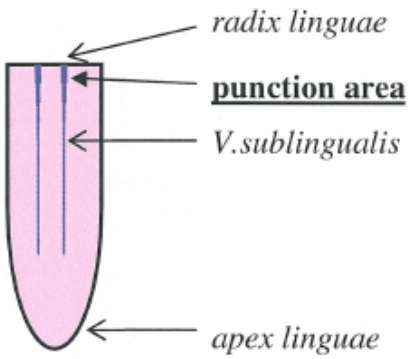


Figure 8: Lower surface of the sublingual vein in mice, including the punction area

Preliminary tests showed that the mouse has to be held in a horizontal position during the blood collection. This prevents any aspiration of blood during the sampling. If the animal is brought in at a steeper angle to the micro tube, the blood that fills the mouth could reach the nasal respiratory tract, with the risk of consequent dyspnoe. The proper way to hold the mouse during the blood collection is shown in Figure 9.

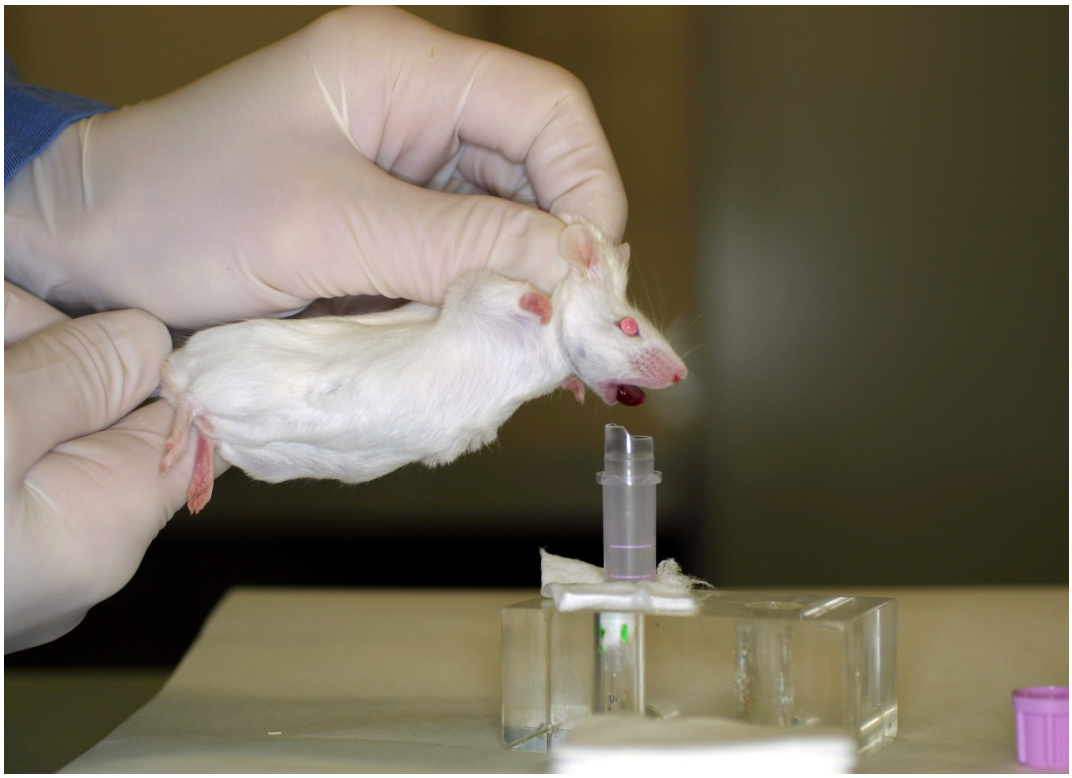


Figure 9: Holding and positioning of a mouse during sublingual blood collection

All of the mice survived the procedure (general anesthesia and blood sampling) without incidents, except for one mouse that did not recover from the anesthesia.

7.1.2.2 Blood collection from the retrobulbar venous plexus

300µL of blood was collected using the retrobulbar venous plexus method. All of the animals survived the collection procedure.
In some cases the blood did not fill the capillary immediately after its insertion. However, the punction of the retrobulbar venous plexus was successful after additional rotation of the capillary.
Bleeding from the punction channel outside and parallel to the capillary was seen as another complication in a few cases.
The collection procedure lasted 20–30 seconds.

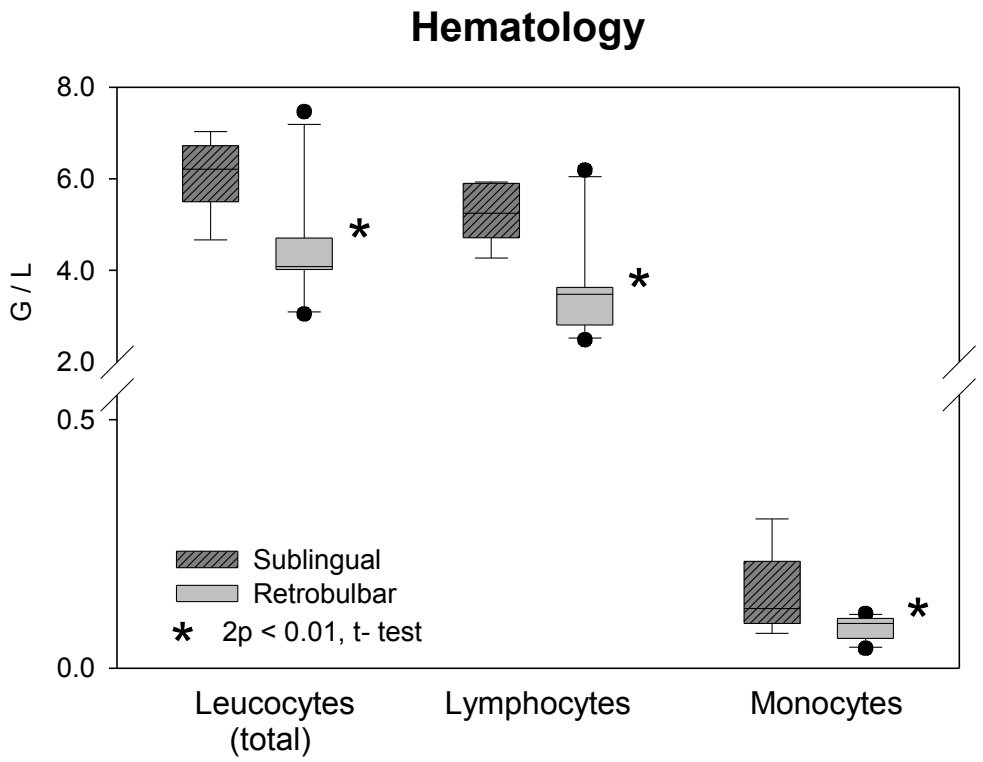
7.1.3 Investigation of lingual damage

The visual inspection of the sublingual area of the mice from the pilot study was performed while the animals were once again under anesthesia.
During checks made 2 hours and 3 days after the blood sampling, no symptoms were apparent in any of the mice.
An additional group of 9 mice was investigated. Among these 9 animals, 2 showed small hemorrhages next to the punction site. A small spot the size of a pinhead was found in one animal, and the other animal had an area of redness approximately 1mm in size parallel to the punctured vein.

7.1.4 Hematology and clinical chemistry

The hematology examination included counts of the total white blood cells, differential white blood cells (WBC), red blood cells, hemoglobin, hematokrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and platelets. Alanin aminotransferase, albumin, alkaline phosphatase, aspartate aminotransferase, glucose, triglyceride, urea, calcium, chloride, potassium, sodium and total protein were defined as the clinical chemistry parameters.
In a comparison of the hematology and clinical chemistry parameters of blood collected using the sublingual and the retrobulbar technique respectively, significant differences were found between the white blood cells (WBC), monocytes, lymphocytes, aspartate aminotransferase (AST), alanin aminotransferase (ALT) and glucose.

Hematology
Statistically significant differences between the hematology parameters of the pilot study and the main study were combined and presented in Figure 10.



Explanation of illustrated box plots:

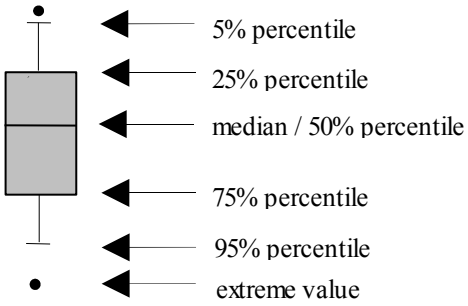


Figure 10: Statistically significant blood values – effects on hematology

Sublingual blood yielded higher values for white blood cells (WBC) and lymphocytes than blood that was taken from the retrobulbar venous plexus. The WBC and lymphocyte values of the retrobulbar blood samples showed a wider distribution illustrated by larger whiskers. In addition, extreme values lay outside the whiskers.
Monocyte values for the sublingual group showed a wider distribution and also higher values than the monocyte ranges of the retrobulbar group. Unlike the values for the monocytes in the retrobulbar group, there were no extreme values for the monocytes in the sublingual group.

There were differences between the groups used in both parts of the study with regard to the incidence of clots. The hematology results for the pilot study showed clots in 1 out of 9 samples. By contrast, the hematology samples of the main study contained clots in 8 out of 10 samples taken from the sublingual and also the retrobulbar group. Coagulations were also seen in the blood smears. The procedures used for sampling and for handling the micro tubes after collection were the same as the procedures used for earlier blood collections.

Clinical chemistry
Statistically significant differences between the clinical chemistry parameters of the pilot study and the main study were combined and presented in Figure 11.

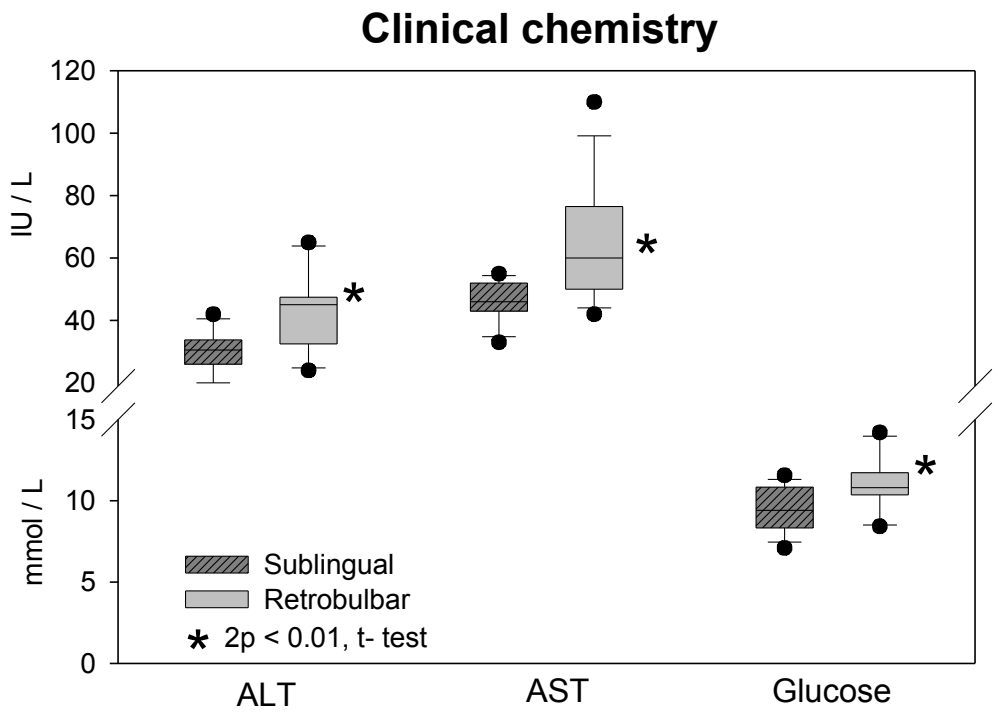


Figure 11: Statistically significant blood values – effects on clinical chemistry
Legend: ALT = alanin aminotransferase; AST = aspartate aminotransferase
Explanation of illustrated box plots is given in the legend of Figure 6

The values for aspartate aminotransferase (AST) and alanin aminotransferase (ALT) in the retrobulbar group are higher and have a wider distribution than the sublingual group. In contrast to the AST and ALT ranges for the sublingual group, extreme values are seen in these parameters of the retrobulbar group.
The glucose values for blood collected using the retrobulbar method are higher, but the range is narrower than the glucose values for sublingually sampled blood. The glucose values for the retrobulbar group show a higher statistical spread within larger whiskers than the glucose box plot of the sublingual group.

7.1.5 Ophthalmoscopy

Physical or functional changes were detected in 4 out of 9 mice (44%) in the pilot study and in 23 out of 30 mice in the main study (77%). The details are shown in Table 4.

Table 4: Ophthalmoscopy findings and numbers of affected animals in both parts of the study

	Examination before blood collection*		2h after blood collection**		1 day after blood collection*		3 days after blood collection*	
Study part	**Pilot n=9**	**Main n=30**	**Pilot n=9**	**Main n=30**	**Pilot n=9**	**Main n=30**	**Pilot n=9**	**Main n=30**
Symptom								
Red nasal discoloration (conjunctives)			2	2			2	
Swelling (conjunctives)							1	
Discharge, white					1			
Slight overflow of tears				3	1			
Persistent bleeding				3				
Reddened sclera				2				
Slight opacity of cornea					1			
Moderate opacity of cornea					1			
Bleeding into anterior chamber of the eye				1				
altered visual axis					1		1	
Iris not circular (dyscoria)			1	1				
Iris not apparent			1					
Reddened iris							1	
Invagination of blind spot						1		1
Reduced bulbus size						1	1	
Photophobia / fundus examination impossible						1		
No miosis				5				
Limited/delayed miosis (before mydriatic drops were used)		2	1	11	1		2	
No/limited mydriasis (after mydriatic drops were used)					1			
Total number of affected right eyes	1	2	4	23	2	6	2	5

Legend: * = including examination without use of mydriatic eye drops followed by examination using mydriatic eye drops
** = no use of mydriatic eye drops to prevent additional damage to eye structures

In Table 4 every symptom that occurred in an animal was listed. Therefore the number of detected symptoms is not equal to the total number of affected right eyes.
Tissue damages or symptoms (e.g. discharge, nasal discoloration of the conjunctives or sclera, opacity of cornea, dislocation of pupil, reduced bulbus, persistent bleeding) and dysfunctions of the iris (delayed or missing miosis or mydriasis) in the punctured right eyes were found. The examination that was carried out 3 days before the punction yielded no findings in both study parts with the exception of two mice in the main study. The two mice showed delayed miosis of the right eye in this examination.

Most symptoms were found two hours after the blood collection, and fewer symptoms were observed a day after the collection. Missing or delayed miosis played a role in 16 out of 30 mice in the main study two hours after blood had been collected from the right eye.
Three days after the blood collection the number of findings decreased rapidly.

Even though the non-punctured left eyes were used as control tissue, unexpected symptoms were found in three animals (data were not presented in Table 4). Symptoms were found during the examinations carried out 3 days before and 2 hours after the punction of the right eye. One mouse attracted attention because of the altered visual axis of the left bulbus three days before and 2 hours after punction of its right eye. This symptom was not seen 1 and 3 days after the punction.
Two mice showed no or delayed miosis, reddened sclera or a red discharge during the same time span.

7.1.6 Histopathology

No macroscopic findings were noted in the pilot study or the main study with the exception of one animal that did not recover from anesthesia. A focal dark red discoloration was macroscopically seen. This discoloration was diagnosed as a focal hemorrhage in the subsequent histopathology examination.
The histopathology results of the sublingually punctured mice in the pilot and main study are shown in Table 5 and 6.

7.1.6.1 Microscopic results of the pilot study / Sublingual vein punction:

Lesions were found in 6 out of 9 sublingually punctured animals of the pilot study. The slides showed destruction of muscle fibrils of the tongue and accumulation of erythrocytes in the affected animals. The muscle cells of the affected areas were brightened and surrounded by phagocytes. The focal accumulations of erythrocytes were localized in the submucosa or the muscular tissue respectively.
The evaluated grade of severity was 1 (the key for evaluated grades of severity is given below Table 6).

7.1.6.2 Microscopic results of the main study / Sublingual vein punction:

The punction of the sublingual vein causes minimal focal injury in the form of destruction of muscle fibrils of the tongue muscle and focal accumulation of erythrocytes in the submucosa. The muscle cells of the affected areas were brightened and surrounded by phagocytes (mostly lymphocytes and macrophages).
Lesions were induced in 15 out of 30 sublingually punctured mice in the main study.
The evaluated grade of severity was 1.1.

7.1.6.3 Microscopic results of the pilot study / Retrobulbar venous plexus punction:

Lesions were found in 8 out of 9 animals punctured using the retrobulbar method in the pilot study.
The microscopic slides of the eye region showed lesions that involved adnexa structures of the eyes (Harderian gland, eye muscle tissue, optic nerve).
The muscular lesions were minimal to moderate and included destruction of the muscular structure and focal accumulations of erythrocytes. Infiltrations by phagocytes (mostly lymphocytes and macrophages) were seen in the affected areas.
The medial Harderian gland lost its glandular structure in the affected areas. The changes were characterized by vacuolar degeneration, infiltration of phagocytes and hemorrhage. One animal showed diffuse changes of the medial and also the lateral part of the Harderian gland.
A focal lesion was noted in the optic nerve of one animal. The affected area showed vacuoles and lucency of the tissue.
The evaluated grade of severity was 1.7.

7.1.6.4 Microscopic results of the main study / Retrobulbar venous plexus punction:

Lesions were induced in 28 out of 30 sublingually punctured mice in the main study. The microscopic slides of the eye region showed changes that involved adnexa structures of the eyes (Harderian gland, eye muscle, optic nerve).
Lesions of the eye muscle tissue were minimal to moderate. Destruction of muscle fibrils with infiltration of lymphocytes and macrophages was noted. Some slides showed focal hemorrhage in the muscle tissue.
Harderian gland tissue showed minimal to marked focal lesions with the loss of glandular anatomy and the development of vacuoles as a result of cell destruction. Infiltrations of phagocytes or focal hemorrhage were noted.

As seen in the exploratory study as well, a focal lesion was seen in the optic nerve of one animal. Nervous cells of the affected area were brightened as a result of the early stages of destruction. The development of vacuoles was also noted.
The evaluated grade of severity was 1.5.

The key used to evaluate the grades of severity was in accordance with standard common practice at Novartis Toxicology, Switzerland and is given in the legend below the tables of histopathology results.

Table 5: Histopathology results of sublingual blood collection

Sublingual punction	Injury /n Mean severity	hemorrhage /n Mean severity	Total of affected mice (%) Mean severity of affected mice
Pilot study	5/ 9 1	2/9 1	6 (67%) 1
Main study	6/30 1	13/30 1.2	15 (50%) 1.11

Table 6: Histopathology results of retrobulbar blood collection

Retrobulbar punction	Injury to eye muscle /n Mean severity	hemorrhage of muscle /n Mean severity	Lesion of N.opticus /n Mean severity	Harderian gland atrophy /n Mean severity	Harderian gland necrosis /n Mean severity	Total of affected mice / (%) Mean severity of affected mice
Pilot study	3/9 2	5/9 1.4	1/9 1	3/9 1.7	5/9 2	8 / 89% 1.7
Main study	12/29* 1.3	3/29* 1.3	1/26* 1	3/30 1.7	21/30 2	28 / 93% 1.5

Legend: * = Although 30 mice in the retrobulbar group of the main study were examined, in a few slides some tissues could not be shown. Therefore the examination of tissue in these slides was not possible.

Key for evaluated grades of severity:

Grade of severity	Percentage of affected organ area in histological slide
1	12.5%
2	25%
3	50%
4	75%
5	100%

7.2 Guinea pig

The experimental punction of the sublingual vein in both guinea pigs was not successful. The section of the mouth region of both guinea pigs showed a different anatomy of the tongue compared to other small rodents. The tongue of a guinea pig consists of two sections: the apex of the tongue that is free to move and the immovable corpus of the tongue that is fixed to its base with connective tissue. An eminent *torus linguae* of the tongue is found in the caudal area of the *corpus linguae* (see Annex, Figure 19).
Figure 12 shows a section of the lower surface of a guinea pig's tongue. A sublingual vein is not visible.

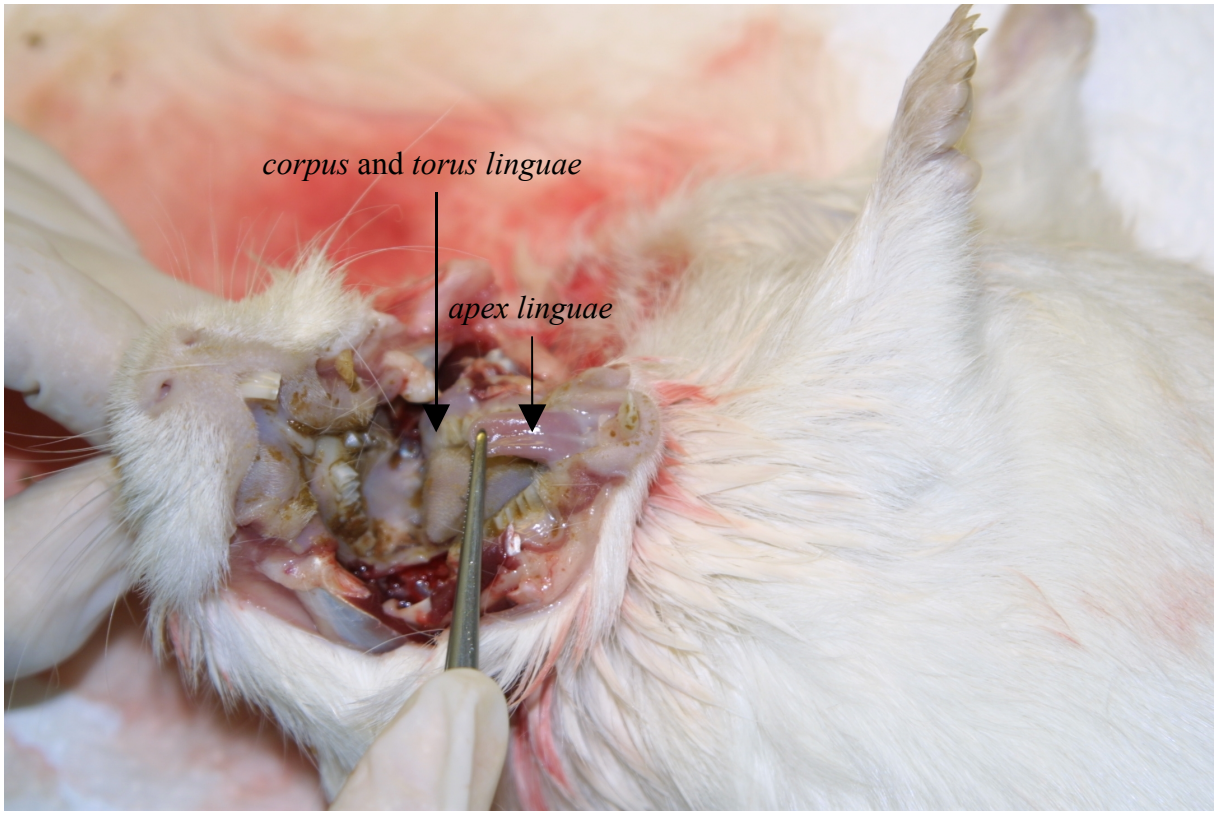


Figure 12: Section of the lower surface of the tongue of a guinea pig

The histological slides did not show the existence of a sublingual vein in guinea pigs. The slides only showed the occurrence of a small profound lingual vein. Figure 13 shows a tongue of a guinea pig in transversal section. On the sublingual surface of the tongue no sublingual vein is visible. Figure 14 illustrates the tongue in longitudinal section. Only a small profound vein is illustrated, but no sublingual vein can be seen.

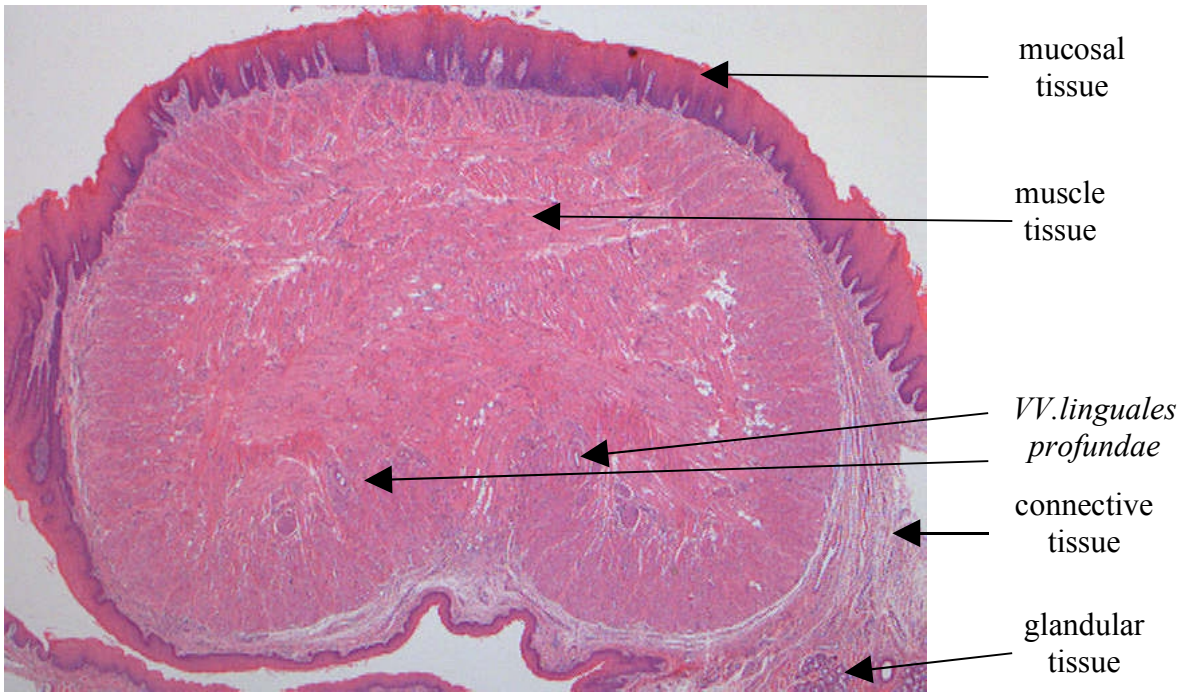


Figure 13: Tongue of a guinea pig in transversal section between corpus and apex

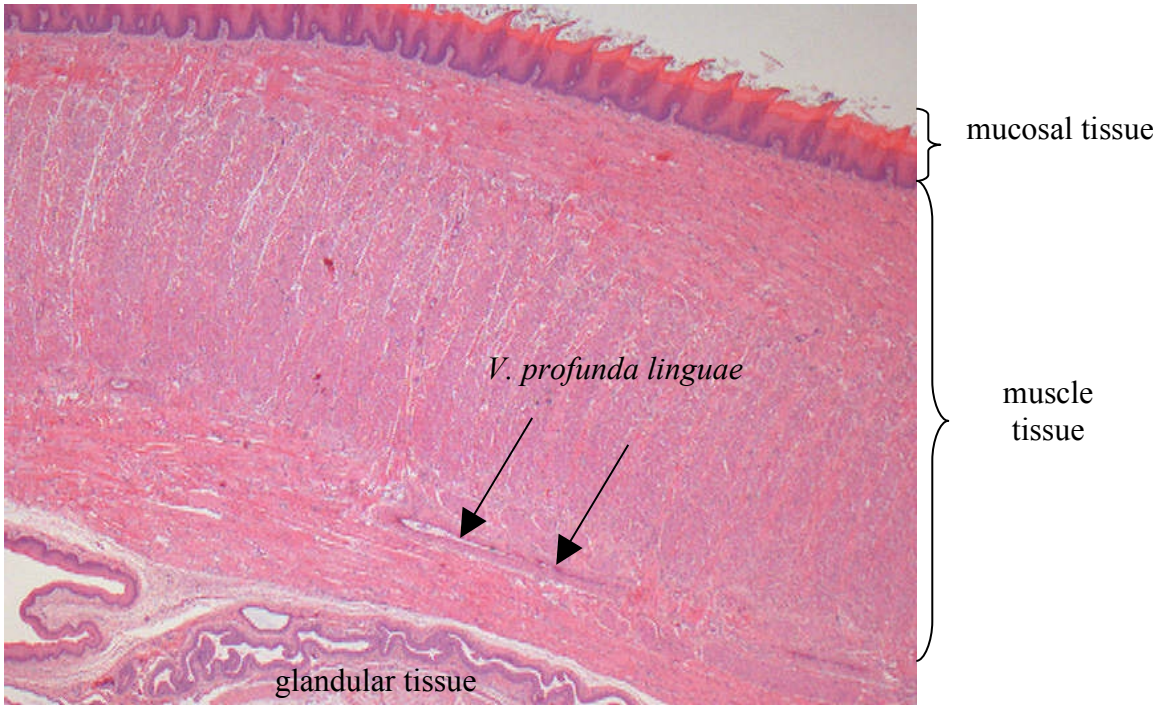


Figure 14: Tongue of a guinea pig in longitudinal section

7.3 Hamster

Blood was collected without problems from every hamster. The procedure was done easily and rapidly. The blood collection lasted 10–20 seconds. The punction of 10 hamsters including anesthesia was completed in an overall time of 20 minutes.
The oral cavity with the tongue and sublingual veins is wider than the oral cavity of other species. The sublingual vein has a larger diameter and the vein is very clearly visible.
Up to 1cm of the vein can be punctured (with individual differences). The ideal punction area is illustrated in Figure 15.

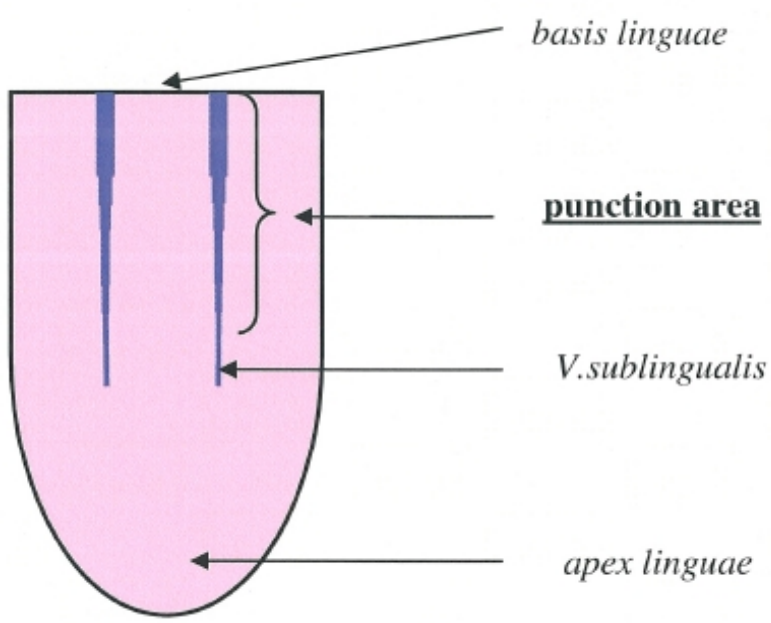


Figure 15: Illustration of the lower surface of a hamster's tongue

Attention has to be paid to make sure the tongue is immobilized properly. The tongue's mucosa is made very slippery by saliva and the surface tissue is quite compact. For this reason, the tongue has to be tightly fixated in order to prevent it from sliding backwards away from the needle at the beginning of the punction.
The tongue is easily movable within the oral cavity. It can be restrained more effectively by pressing it against the lateral side of the upper incisors.
No complications arose during the blood collection procedure.
No continuing bleeding was noted.
The individual results of the hematology and clinical chemistry examinations are summarized in the Annex (see Tables 11 and 12).

8 Discussion

8.1 Mouse

The laboratory mouse is the most frequently used animal model in preclinical pharmacological or toxicological studies. During these studies the examination of blood to determine the different parameters of drug metabolism plays a major role (SUCKOW et al., 2001). Numerous blood sampling techniques for mice are available. Many of these methods are unsatisfactory with regard to their practicability, the volume of blood that is collectable per punction, or the potential for severe tissue damage. Furthermore, some techniques are permitted as terminal methods only, and this limits their use, even though the collectable amount of blood is seen as satisfactory.
The punction of the retrobulbar venous plexus is one commonly used technique for collecting blood from mice and other laboratory rodents. This technique is efficient for collecting large volumes of blood but it has the potential to cause severe tissue damage, particularly to the eye's adnexa structures (eye muscles, Harderian gland, and optic nerve).
ZELLER et al. developed a new method for blood collection from rats that allows multiple repeated blood collections in kinetic studies as well as the efficient collection of large volumes without causing severe tissue damage or influencing the animals' behavior and ingestion (ZELLER et al., 1998). Today this new method of punction of the sublingual vein is recommended for rats in Switzerland, whereas the retrobulbar method is not (Bundesamt für Veterinärwesen, BVET – Richtlinie 3.02).
Knowledge of the advantages of sublingual blood sampling in rats led to the schedule of the present study. The purpose was to apply the technique of taking blood samples from the sublingual vein to mice. First the technique had to be established. Then the retrobulbar and the sublingual methods were compared. The aim of this comparison was to answer the question of whether the sublingual technique in mice yields comparable volumes of blood while having less potential for causing tissue damage, pain and distress in the animals.
Blood sampling from the sublingual vein in mice is suitable but different from the way the method is applied to rats or hamsters. The technician has to take care to follow the correct procedure during the sampling in mice. As shown in preliminary tests, only the caudal part of the sublingual vein has a large enough diameter to permit successful punction. If the vein is punctured closer to the tongue's apex, haematoma and swelling may result as a consequence of insufficient blood flow. During the collection the mouse has to be positioned strictly horizontally; otherwise, it could aspirate the blood that fills its mouth. In this case dyspnoe or even gasping could result. Furthermore, the grasp of the animal's neck skin in order to ensure venous congestion should not be too tight. This could lead to an inadequate blood flow and prevent successful blood collection.
Holding the mouse headfirst before the neck skin is grasped is also different from the way rats are handled during the sublingual blood sampling technique (due to the larger total blood volume in rats this handling is not necessary in this species).
In summary, use of the sublingual method of blood sampling for mice seems to require more practice than its use for rats. Even though the technique is simple, it seems to be easier to learn using a larger species.
The technique permitted the collection of 300µL of blood per mouse. The sublingual method of blood collection was carried out without complications, with the exception of one animal that did not recover from anesthesia. This death was related to the anesthesia.
In preliminary tests the punction of the sublingual vein yielded maximum collectable blood amounts of 1.2mL in mice that were scheduled for euthanasia. For this reason, this method is probably suitable as a terminal technique if exsanguination is required.

In the main study, blood collection from the retrobulbar venous plexus was carried out by an experienced technician. An amount of 300µL of blood per mouse was collected. No severe complications were noted. In some cases blood did not fill the capillary immediately after its insertion. Punction of the retrobulbar venous plexus was achieved through further rotation movements of the capillary around its axis.
Another complication in a few cases was the occurrence of bleeding from the punction route parallel to the capillary. It can be assumed that this was due to obstruction of the microcapillary.
Both techniques were very quick and lasted only 20–30 seconds.
Unlike the retrobulbar technique, a recovery period of two weeks does not seem to be necessary after sublingual blood sampling (Bundesamt für Veterinärwesen, BVET – Richtlinie 3.02). Therefore blood collections from the same site at intervals shorter than two weeks appear to be possible, especially if the puncturing technician is experienced in this procedure. Another advantage is the fact that the sublingual vein is visible in mice. Unlike retrobulbar punction, the sublingual vein is punctured directly. A total of 4-6 punctions using both veins seems to be realistic. Therefore the method can be used in kinetic studies.
Preliminary tests also showed that sublingual blood collection in mice can be carried out by a single person. The congestion of the head veins is achieved by grasping the animal's neck skin before the punction is performed. This procedure is not as easy for one person to do as it is for two persons, because a single person must first puncture the vein and then grasp the neck, and without congestion the veins are less visible. Nevertheless, it is effective and secure if carried out by experienced persons.
A new blood collection technique that enables the collection of large volumes was recently published (GOLDE et al., 2005). The punction of *V.facialis* (also called mandibular or submandibular punction) permits a collection of 0.2 – 0.5mL per animal. Because salivary glands and lymph nodes cover the vein, these structures were also punctured. The disadvantage of this method is that the salivary glands and lymph nodes are damaged. In addition, it can be assumed that the sample is contaminated with tissue fluid and saliva. If the fur is not shaved, the blood can come into contact with the fur, and that will also contaminate the sample. For all of these reasons, this method is not an ideal technique. The mice used in the investigation of GOLDE et al. were conscious during the procedure. For reasons of animal welfare, this method cannot be recommended.
Compared to this method, the sublingual blood collection technique has a lower potential for causing tissue damage. In addition, possible contamination with fur can be excluded.

The punction of the retrobulbar venous plexus is an efficient method of collecting large volumes of blood, but it can potentially cause severe tissue damage, particularly to the eye's adnexa structures (eye muscles, Harderian gland and optic nerve), as shown by different studies (e.g. DIEHL et al., 2001 and HOFF, 2000).
DIEHL et al. specify potential effects including painful retrobulbar hemorrhage, damage of the optic nerve or other intraocular structures with resulting blindness or damage of the bulbus (DIEHL et al., 2001).
HOFF mentions ocular ulcerations, loss of vitreous humor or keratitis as possible types of damage following retrobulbar blood sampling in mice (HOFF, 2000).
MESSOW et al. record hemorrhages and purulent or purulent-necrotic inflammations of the Harderian gland and muscle tissue showing polymorphonuclear leukocytes with atrophy and fibrosis, as well as purulent processes of the bulbus of the eye in certain cases. In addition, the authors report the occurrence of blindness in 1–2% of the test rats (MESSOW et al., 1980).
The performing technician's degree of experience also has a great influence on the severity of the tissue damage. HERCK et al. demonstrate that a technician with more experience produces less tissue damage than a less skilled technician (HERCK et al., 1998).

The technique and its potential to cause severe tissue damage has been mostly investigated in rats, but not at all in mice.
Therefore in this thesis the retrobulbar and the sublingual blood sampling techniques were compared in order to detect the occurrence of damage to the animals' tongue or eye tissue.
The tissue damage observed as a consequence of blood collection was different in quantity and quality, depending on the technique that was carried out.
Sublingual bleeding caused minimal damage in two animals, specifically a small haematoma in one animal and a small reddened area parallel to the punctured vein in the other. These symptoms were seen one day after blood collection and had disappeared two days after their occurrence. These findings were evaluated as minimal and did not amount to a functional handicap.
By contrast, a lot of physical and functional damage to the eye resulting from the punction of the retrobulbar venous plexus was seen during ophthalmoscopy. Physical lesions in the form of swellings, discharge, reddened tissue, opacity of the cornea or (persistent) bleeding were seen, as were various types of dysfunction – mostly dysfunction of the iris, in the form of missing, delayed or faulty miosis or mydriasis (after mydriatic eye drops were used) and dyscoria of the pupil.
Most of the findings were observed two hours after retrobulbar blood collection was done. The number of findings decreased rapidly one day and three days later respectively, especially in the ophthalmoscopy results of the main study.
A larger number of findings were noted in the ophthalmoscopy of the main study than in the pilot study. This result had not been expected, because the retrobulbar punction in the main study was carried out by a more experienced technician (compared to the retrobulbar technician of the pilot study).
The various types of dysfunction – mostly observed as missing, delayed or faulty miosis or dyscoria of the pupil (no circular pupil) – can be explained as an indirect result of temporary damage or irritation of other structures, e.g. the innervating nerves of *M.dilatator pupillae* (fibers of *N.sympathicus*) or *M.sphincter pupillae* (*N.oculomotorius*). A permanent irritation of the nerve fibers of *N.sympathicus* might lead to permanent activity of the *M.dilatator pupillae*. In this case an animal would show missing or faulty miosis. This assumption is also found in the literature. GUM reports on miosis, conjunctival or iridal hyperemia as a result of local irritation (GUM, 1991).
In most cases this dysfunctional miosis of the iris had disappeared a day after blood collection.
The altered visual axis of the right bulbus that was seen in one animal of the pilot study can be evaluated as direct damage to, or indirect irritation of, the eye bulbus muscles. For instance, extensive bleeding might have led to pressure on muscular or nerve tissue resulting in higher tension (*M.rectus ventralis*) or contrary higher dilatation (*M.rectus dorsalis*) of an eye bulbus muscle. This finding occurred after blood was collected, but had not been noted before. For this reason a congenital genesis can be excluded.
Retrobulbar bleeding did not seem to damage the bulbus of the eye directly, even though a reduced bulbus was observed in one animal. This finding could not be confirmed through a histopathology examination. Swelling of the surrounding tissue might possibly lead to inward movement of the bulbus, which would make the bulbus seem reduced in size.
It is uncertain to what extent mice rely upon the eyes (compared to human). But the eye generally is a sense organ in mammalian and it can be expected to be important and sensitive in mice, too. The clinical comparison of physiological damages between the eye and the tongue seems to be justifiable.

The different rates of incidence of punctured tissue due to the sublingual and the retrobulbar punction method respectively can be seen in the pathology examination of the tongue and eye region as well.
The nature of the lesions noted in the pilot study and the main study were similar. There were differences in the severity of the tissue damage, especially in the retrobulbar group.
In the pilot study, 6 out of 9 animals in the sublingual group (= 66.7%) had traumatic lesions of the tongue, while 8 out of 9 animals in the retrobulbar group (88.9%) had lesions of the eye's adnexa structures. Clear distinctions can be noted with regard to the mean severity of tissue damage caused by the two compared techniques: the determined mean severity for the sublingual group of the pilot study was 1.0. By contrast, a mean severity of 1.7 for tissue damage was noted for the retrobulbar group of the pilot study. This is a striking difference from the severity results for the sublingual group.
Because of these findings and contrary to the originally planned schedule, the sublingual and retrobulbar groups in the main study were also subjected to a pathological examination.
In the sublingual group of this part of the study, 15 out of 30 mice (50%) had traumatic lesions of the tongue with a mean severity of 1.1. By contrast, 28 out of 30 animals in the retrobulbar group (93.3%) showed tissue damage to the eye's adnexa structures with a mean severity of 1.5. The mean severity of the injuries in the retrobulbar group is greater than the mean severity of the injuries in the sublingual group, which indicates that a higher grade of severity is caused by this conventional technique. As mentioned above the clinical comparison of physiological damages between the eye and the tongue seems to be justifiable, even though it is uncertain to what extend mice rely on their eye sense.
Nevertheless, if the mean severity for the retrobulbar groups in the pilot study and the main study are compared, a clear difference can be seen between the two values. The punction of the retrobulbar venous plexus was carried out by skilled technicians, because the author lacks the necessary experience to do a skilled punction that minimizes the inevitable tissue damage. For retrobulbar punction in both parts of the study, a highly experienced technician had been scheduled, but this technician was unexpectedly absent in the pilot study. Ultimately a less experienced technician carried out the retrobulbar punction in the pilot study. The retrobulbar punction in the main study was done by the experienced technician as originally planned. Both technicians used the same material and technique. Therefore the clinical comparison of severity grades between the pilot and the main study part seems to be justifiable, even though the technicians' grade of experience differs.
This change of schedule provides additional information about the influence of expertise on retrobulbar blood sampling. The less experienced technician caused tissue damage that was characterized by a higher grade of severity than the tissue damage caused by the more experienced technician. This corresponds with the study of HERCK et al. that was mentioned above (HERCK et al., 1998).
The percentage of affected animals in the group of animals punctured by the retrobulbar method in both parts of the study is similar.
In both parts of the study, the optic nerve of one animal showed a focal lesion characterized by demyelinization with myelinophagocytosis and astrocytosis. This tissue damage is assessed as a consequence of the insertion of the glass microcapillary by mistake. The diagnosis of congenital hypoplasia of the optic nerve, which is extremely rare in rodents and leads to blindness or restriction of the function of the eye, can be excluded. In cases of the occurrence of this congenital abnormality, other structures of the eye show anomalism as well (e.g. malformations of the retina or bulbus). In addition, the congenital hypoplasia of the optic nerve of rodents is not characterized by inflammation. By contrast, the alterations of the optic nerve in the affected mice used in this study show a great number of macrophagocytes and other phagocyte cells.

From the point of view of pathology, the method of sublingual blood collection is suitable in mice. This evaluation is based on the lower grade of severity and the smaller number of affected animals in the sublingual punction groups in both the pilot study and the main study. This is analogous to the results of the other investigations in mice cited in this study.

Different investigations demonstrate that the particular blood collection method used has an influence on the hematology and clinical chemistry of the blood collected.
SCHNELL et al., 2002) assess significant differences between the clinical pathology parameters of blood collected from mice using either the heart, the *V.cava* or the retrobulbar venous plexus as the source. Blood taken from the retrobulbar venous plexus showed an increase of erythrocytes, hemoglobin and hematokrit compared to the other sources. In addition, measurements of enzyme activity, especially of aspartate aminotransferase, increased compared to blood collected using other techniques. The increase of aspartate aminotransferase is explained as a result of tissue or blood cell damage caused by the retrobulbar method (SCHNELL et al., 2002). This result corresponds to the evaluations of MAHL et al., who compared the retrobulbar versus the sublingual blood sampling technique in rats (MAHL et al., 2000). In this study the rats punctured using the retrobulbar method showed an initial decrease of leukocytes (especially of lymphocytes) and higher activities of creatin kinase and aspartate aminotransferase compared to the parameters of sublingually collected blood. The authors reason that the technique of blood collection from the retrobulbar venous plexus results in more severe tissue damage than the punction of the sublingual vein.
As a consequence of differences in specific blood parameters evaluated by various studies, the present study compared the hematology and clinical chemistry parameters of blood taken either from the sublingual vein or the retrobulbar venous plexus.
As shown in the chapter on results, significant differences between the figures for white blood cells, lymphocytes, monocytes, aspartate aminotransferase, alanin aminotransferase and glucose were found in the comparison of the sublingual and the retrobulbar group.
Blood collected from the sublingual vein showed higher values for white blood cells, lymphocytes and monocytes when compared to blood collected from the retrobulbar venous plexus.
White blood cells in general, and especially lymphocytes as well as monocytes, are eminent components of the immune defense system. The oral cavity features wide areas of lymphatic tissue (Waldeyer's tonsillar ring) and is one of the first contact points of antigens affecting the body. Disarming orally ingested antigens is the main function of this lymphatic tissue.
Against this background, it is not surprising that the numbers of white blood cells in the blood collected from the veins of the sublingual region are higher. Hypothetically, a locally increased concentration of these immune defense cells can be assumed. The retrobulbar tissue is insulated, and there is no significant lymphatic tissue behind the bulbus. Nevertheless, the anterior corneal side of the bulbus, together with the eyelids and the lachrymal glands, is drained well and is also a first contact point for outside antigens.
Veins are accompanied by lymphatic vessels, while the venous plexus is not. This aspect is the background of another hypothesis, which explains the different blood parameters according to the different techniques used. If the sublingual vein is punctured, the punction of the lymphatic vessel cannot be avoided. This could result in higher values for lymphatic cells in sublingual blood. Lymphatic vessels are not found next to the venous plexus. The lower values for white blood cells, lymphocytes and monocytes in retrobulbar blood might result from this anatomical fact.
During retrobulbar punction different tissues (conjunctives, bulbus muscles and Harderian gland) were damaged before the venous plexus is punctured, what is different to the sublingual technique. Sublingual blood collection exclusively causes damages of the vein itself or tongue muscle in single cases. The wider statistical spread of white blood cells and

lymphocytes found in blood using retrobulbar technique probably results from the damage of a larger number of different tissues.

Blood collected from mice has a special ability to coagulate easily (NOLAN, 2005). In samples taken during the preparatory tests and the pilot study, clots appeared in an average of 1out of 9 samples. By contrast, hematology samples taken during the main study contained clots in 8 out of 10 samples from the sublingual and also from the retrobulbar group. Clots also appeared in blood smears. The procedures used for the blood sampling and the handling of micro tubes after collection were the same as the procedures used in earlier blood collections. Nevertheless, a systematic error can be assumed.
It is not possible to make a statement about platelet counts. Because both blood sampling methods are concerned, the possibility that the specific technique used has an influence on blood coagulation can be excluded.

The clinical chemistry parameters showed differences between the values for alanin aminotransferase (ALT), aspartate aminotransferase (AST) and glucose that were evaluated as statistically significant. Enzyme activities and glucose values were lower if the blood was collected from the sublingual vein compared to the retrobulbar venous plexus. AST and ALT are organically unspecific cellular enzymes.
ALT is found in the cytoplasm, and activity values increase immediately if cells are damaged. The measure of the increase of ALT activity does not provide any information about the grade of severity of tissue damage (KRAFT and DÜRR, 1999).
AST has different activity values in the tissues of different organs, especially in heart and skeletal muscle. This enzyme is contained in mitochondrions and cytoplasm. For these reasons this parameter is well suited to be a serum indicator of damage to muscle tissue, especially in the case of muscle necrosis. On a small scale, activity values already increase if muscle cell membranes are affected (KRAFT and DÜRR, 1999).
The increases of AST and ALT in blood collected from the retrobulbar venous plexus probably originate from cellular damage to the retrobulbar tissues and conjunctives that are penetrated by the microcapillary during the blood collection process. By contrast, the values for sublingually collected blood are lower because only mucosal surface tissue, the vein itself and, in some cases, the tongue muscle are affected.
During retrobulbar punction different tissues (conjunctives, bulbus muscles and Harderian gland) were damaged before the venous plexus is punctured, what is different to the sublingual technique. Sublingual blood collection exclusively causes damages of the vein itself or tongue muscle in single cases. The wider statistical spread of AST and ALT found in retrobulbar collected blood probably results from the damage of a larger number of different tissues.
As mentioned above, the single measurement of the increase of ALT activity does not give any information on the grade or severity of tissue damage, but combined with the increase in AST activity, it seems acceptable to conclude that more severe tissue damage is caused by retrobulbar punction.
Blood obtained by the retrobulbar method contained higher values of glucose than blood from the sublingual vein. This difference is significant but not conspicuous in the way the enzyme activity values are. As a substance that is essential for cell functions and metabolism, glucose is an important component of muscle tissue. If muscle tissue is damaged, glucose is released and serum glucose values increase by analogy.
During the punction of the retrobulbar venous plexus, damage to eye muscle tissue cannot be definitely avoided. The Harderian gland and the conjunctive structures were also damaged and it can be assumed that this was the source of glucose release.

Ideally, only the vein is punctured during the sublingual punction but the subjacent tongue muscle is not. This may explain the lower serum glucose levels of blood obtained by the sublingual method. The wider statistical spread of sublingual blood glucose may have been caused by accidental single punctions of the subjacent muscle tissue.
Glucose is a specific parameter that increases in stress situations. It is doubtful whether the handling of the unconscious mouse during the blood collection is more stressful, resulting in an increase of glucose.

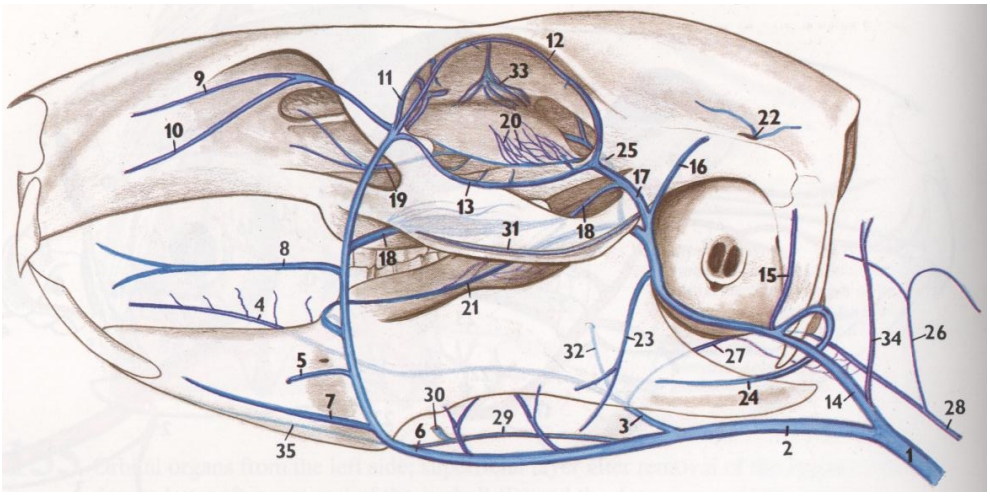
In 2000 MAHL et al. compared the influence of retrobulbar and sublingual blood collection in Hanover rats on body weight, food and water consumption (MAHL et al., 2000). The authors detected no difference between the two techniques in terms of their influence on these parameters. The investigation of MAHL et al. corresponds to the results of the present study. The monitoring of the individual animals showed no statistical differences between the body weight or food and water consumption of the sublingual group compared to the retrobulbar group.
In terms of food consumption, all groups showed an unexpected increase of curves starting on day 5. The food consumption returned to the normal standard on day 8. Although the decrease of food consumption on day 7 in both of the punctured groups was more apparent, it was not statistically significant. A slight influence of the blood collection might be assumed.
However, the food consumption in all groups increased after the rodent food was removed and new food was given from the same batch on day 5. This might explain the change of food consumption in both the punctured and the non-punctured animals. According to this theory, the food consumption should have increased again as a result of the next change of food on day 12. But this effect was not noticed. Therefore the reason for the changed shape of all curves cannot be explained for sure.
In addition, a significant variance between the food consumption of the two control groups was seen on day 9. This difference might be explained as a result of the greater decrease of food consumption in the punctured animals that had to be compensated for.
No statistically significant variances were seen in the water consumption of the sublingual, retrobulbar and control groups during most of the investigation. Apart from this, both control groups showed significantly higher water consumption on day 9 than did the sublingual or the retrobulbar group. A statistical significance was also noticed on day 11 in control group I (which was neither exposed to blood collection nor anesthesia). Irrespective of this, no difference was observed between the water consumption of the different groups of punctured mice (independent of the blood collection technique). As shown by the water – food consumption ratio, the water consumption of mice in all groups was normal.
In conclusion, no influence of the respective blood collection techniques on body weight or food and water consumption was noticed. Apart from this, the loss of blood itself and the required anesthesia influenced these parameters. But these influences were the same, irrespective of the technique used.

In conclusion, punction of the sublingual vein can be recommended as a suitable technique for mice. The technique is simple, fast and effective. 300µL and more can be collected either by one or two persons using this method.
The method permits the collection of large volumes of blood. Like the conventional retrobulbar technique, it does not have any influence on the animals' body weight or food and water consumption. Another advantage of sublingual punction is the fact that it causes less tissue damage. Therefore it can be expected that it will have fewer adverse effects on the animals' well-being.
As with the retrobulbar method, blood collection from the *V.sublingualis* requires experience. The procedure is not as easy to learn for mice as it is for rats.

If sublingual blood collection in mice is carried out less than optimally, an unsatisfactory blood collection will probably result. However, it will not result in severe tissue damage, which is known to be the case with retrobulbar blood sampling under comparable conditions. Significant differences were noticed in the blood parameters, depending on the blood collection technique that was used. This aspect should be kept in mind if investigations are scheduled using this blood collection technique for mice. New reference values are recommended if the new sublingual method is used for mice.

8.2 Guinea pig

Sublingual veins do not exist in guinea pigs.
The small diameter of the *V.lingualis* and the *V.profunda linguae,* as well as their location deep below the surface, as shown by the macroscopic examination of the tongue and the histopathology, do not permit a successful blood collection in guinea pigs.
This result corresponds with illustrations of the anatomy of the veins of the head of the guinea pig. As shown in an anatomical illustration of POPESKO et al., the guinea pig possesses only the *V.lingualis* and the deep *V.profunda linguae*, but no sublingual vein (POPESKO et al., 1992). This is illustrated in Figure 16:



1 *v. jugularis externa* – external jugular vein
2 *v. linguofacialis* – linguofacial vein
3 *v. lingualis* – lingual vein
4 *v. profunda linguae* – deep vein of tongue
5 *ramus foraminis mentalis* – branch of mental foramen
6 *v. facialis* – facial vein
7 *v. labialis inferior* – vein of lower lip
8 *v. labialis superior* – vein of upper lip
9 *v. dorsalis nasi* – dorsal nasal vein
10 *v. lateralis nasi* – lateral nasal vein
11 *v. frontalis* – frontal vein
12 *v. palpebralis superior* – upper palpebral vein (vein of upper eyelid)
13 *v. palpebralis inferior* – lower palpebral vein (vein of lower eyelid)
14 *v. maxillaris* – maxillary vein
15 *v. auricularis caudalis* – caudal auricular vein
16 *v. auricularis rostralis* – rostral auricular vein
17 *v. temporalis superficialis* – superficial temporal vein
18 *v. buccalis* – buccal vein
19 *v. infraorbitalis* – infraorbital vein
20 *plexus ophthalmicus* – ophthalmic plexus
21 *v. profunda faciei* – deep facial vein
22 *v. emissaria meatus temporalis* – emissary vein of temporal meatus
23 *ramus massetericus* – masseteric branch
24 *v. massetericus* – masseteric vein
25 *v. ophthalmica externa dorsalis* – dorsal external ophthalmic vein
26 *v. occipitalis* – occipital vein
27 *ramus communicans* – communicating branch
28 *v. jugularis interna* – internal jugular vein
29 *v. sublingualis* – sublingual vein
30 *arcus hyoideus* – hyoid arch
31 *v. transversa faciei* – transverse vein of face
32 *v. alveolaris inferior* – lower alveolar vein
33 *plexus orbitalis dorsalis* – dorsal orbital plexus
34 *ramus parotideus* – parotid branch
35 *v. submentalis* – submental vein

Note:
3: V.lingualis; 4: V.profunda linguae

Figure 16: Head veins of a guinea pig including *V.profunda linguae* and *V.lingualis* (POPESKO et al., 1992)

In conclusion, blood collection from the sublingual vein is not a suitable technique to use for guinea pigs.
This result was not expected and led to further additional search of literature (especially anatomical illustrations as shown in figure 16). The lack of the sublingual vein usable as punction site can be viewed as a disappointment. Although many blood collection techniques are known and used for guinea pigs, none of them is completely satisfactory in terms of simplicity of technique, collectable blood volume or potential for tissue damage.
An alternative method of blood collection for this species would be desirable.

8.3 Hamster

Blood collection from laboratory hamsters is essential and is a major object of investigation in a great number of experimental studies concerning this species. Unfortunately, the range of available blood collection techniques is narrow, and many of these techniques do not permit the collection of large samples without resulting in tissue damage if they are non-terminal at all. A few terminal methods are described, e.g. punction of the abdominal aorta, the *V.cava* or the heart. The use of these techniques is quite limited.
Other techniques are not satisfactory in terms of the collectable amount of blood, e.g. punction of the lateral tarsal vein, *V.saphena* or tail tip amputation. In addition, these methods require experience in order to be done successfully.
The commonly used technique of punction of the retrobulbar venous plexus is satisfactory in terms of the blood volume it yields, but, as shown in rats as well, it is connected with potential tissue damage, especially to the eye's adnexa structures (HERCK et al., 1992). The use of this technique is strictly regulated in Switzerland in order to minimize the severity of damage to the animals in laboratory animal studies (Bundesamt für Veterinärwesen, BVET – Richtlinie 3.02).
In 1998 ZELLER et al. refined the method of blood sampling from the sublingual vein in rats (in former times, blood sampling from this vein was done by incision of the vein) and developed the punction of this vessel (ZELLER et al., 1998). This method enables the sampling of large blood volumes in rats and also permits repeated collections, e.g. in pharmacokinetic studies, without causing unacceptable tissue damage.
The aim of this study was to adapt the sublingual punction technique to the hamster. As shown in the section on methodology, this method is a suitable technique for hamsters and seems to be even easier than the procedure for the rat. The oral cavity of the hamster is wider than that of the rat, and as a corollary the sublingual veins have a larger diameter, a fact that facilitates the punction. Unlike the rat tongue, the hamster's tongue is more slippery because of salivation and more easily movable within the oral cavity. In addition, the mucosal tissue is more compact compared to the rat's mucosa. Therefore more attention has to be paid to restraining the hamster's tongue. Otherwise the punction could fail.
The blood unlikely was contaminated with saliva because the cotton bud absorbed it when the tongue was pulled out of the mouth with rolling movements the cotton bug around its axis.
The collection of 500µL and 750µL respectively was done without problems. The collection of larger amounts seemed to be possible, but was not done because of the permitted maximum volumes per animal.
Because of the facilitated procedure and less potential for tissue damage, this method appears to have the potential to replace the traditional method of blood collection from the retrobulbar venous plexus.
Therefore blood sampling from the sublingual vein can be recommended in hamsters.

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10 Index of illustrations and tables

11 Annex

11.1 Available blood collection techniques in mice, guinea pigs and hamsters

With the exception of the retrobulbar and sublingual techniques, all of the methods that are in use for blood sampling in mice, guinea pigs and hamsters at the moment have been described on the basis of the scientific literature. The techniques for the punction of the retrobulbar venous plexus or the *V.sublingualis* in these species is explained in the introduction of this thesis.
The main explanation concerns the mouse species. Differences in the use of these techniques for guinea pigs or hamsters are mentioned separately.

Many blood collection techniques have been developed for mice, guinea pigs and hamsters. These methods can be divided into groups according to the following aspects:
- Techniques classified as terminal
- Non-terminal techniques yielding small to moderate blood volumes
- Non-terminal techniques yielding moderate to large blood volumes.

Cardiac puncture, decapitation, blood collection from the *V.cava caudalis* (*inferior*), the abdominal aorta or the *V.axillaris* (*Plexus brachialis*) rank among the **terminal techniques**.

Cardiac puncture (cardiocentesis) requires general anesthesia in any case. The unconscious animal is placed on a smooth surface and the punction is carried out using a 24-gauge hypodermic needle.
The mouse is lying on its left side and is punctured between the 5th and 6th rib, with the needle being moved in the direction of the heart (SUCKOW et al., 2001). The procedure is also described in the same way when the mouse is placed on its right side for punction of the left ventricle (Animal Care and Ethics Committee of the University of Newcastle, 1999). Alternatively, the mouse is lying on its back, so that the cannula passes the diaphragm laterally to the xyphoid and medially at a 20–30° angle to the animal's horizontal axis to the sternum in the direction of the heart [24] Institutional Animal Care and Use Committee of the University of Iowa, 2002). TERRIL and CLEMONS recommend the punction of guinea pigs using a 20 to 25-gauge hypodermic needle at a 30 to 45° angle (TERRIL and CLEMONS, 1998).
If this method is used for hamsters, the unconscious hamster is lying on its left or right side and the punction needle is inserted into the 4th to 5th intercostal junction, directly at the apex beat of the heart (FIELD and SIBOLD, 1999). Apart from its risk of mortality and its classification as a terminal procedure, some authors note that another disadvantage of this method is the heart's tendency to roll under the pressure of the exploratory needle (PANSKY et al., 1961).
While collecting blood the technician should carefully aspirate the syringe to facilitate the blood flow. Irrespective of the chosen route of the needle, it is important to collect the blood slowly to avoid the collapse of the heart as a result of overly massive negative pressure.
This technique needs to be carried out by an experienced technician (SUCKOW et al., 2001). Most guidelines (i.e. DIEHL et al., 2001, Bundesamt für Veterinärwesen, BVET – Richtlinie 3.02, FIELD and SIBOLD 1999) evaluate cardiac puncture as a terminal technique by reason of the potential appearance of pneumothorax, cardiac tamponade or cardiac arrest.
In Switzerland this technique is not recommended for mice because of its potential risk for the animal's life or health and the availability of more gentle methods (Bundesamt für

Veterinärwesen, BVET – Richtlinie 3.02). The same guideline recommends that this technique be used in guinea pigs and hamsters. If a second punction of the heart is scheduled, the procedure has to be done as a terminal procedure under general anesthesia in authorized animal experiments in Switzerland (Bundesamt für Veterinärwesen, BVET – Richtlinie 3.02).

Decapitation of a mouse means cutting through the neck near the head using a special sharp instrument (in most cases, a guillotine). The immediate blood loss leads to a massive decrease of blood pressure and therefore unconsciousness of the mouse (Bundesamt für Veterinärwesen, BVET – Richtlinie 3.02).
Like cardiac puncture, the decapitation of mice permits the collection of large blood samples but also demands well-trained personnel. The disadvantage of these techniques lies in the fact that tissue fluid and saliva could possibly contaminate the sampling (SUCKOW et al., 2001).
The advantages of this method are that it is quick, effective and yields large volumes of blood. The collected blood is not influenced by any chemicals (no anesthesia is required).
This technique requires special equipment and may be aesthetically unacceptable for some technicians. If no anesthesia or sedation is used, the Institutional Animal Care and Use Committee of the University of Iowa demands scientific justification [25] Institutional Animal Care and Use Committee of the University of Iowa, 2004).

Blood from the *V.cava caudalis* (*inferior*) can also be collected from an anesthetized mouse placed in a supine position. The abdominal skin has to be disinfected using 70% ethanol. A 2cm longitudinal cut along the median line opens the abdomen. The organs are displaced toward the side. The *V.cava caudalis* is found parallel to the right side of the vertebral column next to the *aorta abdominalis* (ADEGHE and COHEN, 1986).
This procedure is also described for use in hamsters (FIELD and SIEBOLD, 1999).
The *V.cava* is separated from its surrounding fascia. The ideal region for punction is the point of inflow of the left and right *V.iliaca communis* into the *V.cava*. A 23 G “butterfly” needle combined with a 5mL syringe is inserted into the vein within 0.5cm. Blood fills the syringe’s conus immediately, a process that can be facilitated by a light aspiration of the syringe (ADEGHE and COHEN, 1986).

Blood collection from the aorta is similar to the procedure of *V.cava* blood collection. The anesthetized animal is placed in a supine position, the abdomen is opened with a large U-shaped incision and the organs are relocated toward the side. After a vertical incision has been made through the diaphragm, the aorta is visible and can be used for blood sampling (LUSHBOUGH and MOLINE, 1961).
The procedure can also be used for hamsters. FIELD and SIEBOLD recommend a medial incision that opens the abdomen (FIELD and SIEBOLD, 1999).

Blood sampling from the *V.axillaris* (also called *Plexus brachialis*) requires a comparable dissection of an anesthetized mouse that is held in a supine position with its forepaws drawn up. Using a sharp scalpel, a deep cut is made through the skin of the axillary region next to the thorax. Clamps are placed to keep the cut open. Blood can be collected if an incision of the vessel is carried out (HOFF, 2000).
The axillary vein may be covered by *Mm. pectoralis major* and *latissimus dorsi*. The sideward dissection of these muscles permits an easier inspection of the vessels (YOUNG and CHAMBERS, 1973).

By exsanguination 1-2mL of blood can be collected using this technique [24] Institutional Animal Care and Use Committee of the University of Iowa, 2002).
If the animal has not died by exsanguination it has to be killed immediately after the blood collection, e.g. by cervical fracture while still anesthetized (YOUNG and CHAMBERS, 1973) or by cutting through the cervical spine without delay (ADEGHE and COHEN, 1986).

Moreover, the Swiss legislation guideline for euthanasia of laboratory animals recommends cervical dislocation or administration of an overdosed injectable anesthetic (pentobarbital or barbiturate combinations) or carbon dioxide for long-term exposure after breathing has stopped to assure the complete euthanasia of the animal (Bundesamt für Veterinärwesen, BVET- Richtlinie 3.01).

These techniques (cardiac puncture, decapitation, blood collection from the *V.cava caudalis* (*inferior*), abdominal aorta and *V.axillaris*) permit the collection of a large amount of blood. Because these are exclusively terminal methods, their range of application is limited. If non-terminal methods for blood sampling of large volumes are required, other methods have to be scheduled.
Many blood sampling techniques for mice, guinea pigs and hamsters are available that are non-terminal procedures and require less material and time.

If the sampling of only **minimal to moderate blood volumes** is required, the available techniques include tail tip amputation, punction of the lateral tail vein, incision of the lateral tail vein, punction of the *V.saphena*, punction of the lateral tarsal vein, punction of *the foot vein,* punction of the marginal ear vein, punction of the peripheral ear vessels or punction of the dorsal penile vein.

The amputation of the tail tip is carried out with a sharp scalpel. About 0.5mm of the tail tip is clipped. The tail vein is truncated as well; blood can be collected in an amount up to 0.1mL. This method is described differently by different institutions.
The working group of EFPIA (The European Federation of Pharmaceutical Industries and Associations) and EVCAM (European Center for the Validation of Alternative Methods) recommends the ablation of a maximum of 0.5mm of the tail tip. The repeated removal of the clot also allows the repeated sampling of minimal blood volumes. In addition, anesthesia of the animal during this manipulation is recommended (DIEHL et al., 2001).
By contrast, the Institutional Animal Care and Use Committee of the University of Iowa calls the technique of tail tip amputation a non-acceptable method in general [24] Institutional Animal Care and Use Committee of the University of Iowa, 2002).
Swiss legislation does not judge this method as strictly and recommends an exclusive single tail tip amputation as well as subsequent repeatable clot removal (Bundesamt für Veterinärwesen, BVET–Richtlinie 3.02).
This guideline also recommends that this technique be used only one time for hamsters. Blood volumes up to 100µL can be obtained by repeatable removing of the clot.

The punction of the lateral tail vein is suitable in mice if blood volumes of 0.1–0.15mL are needed. This technique requires an adequate restraint of the mouse during the blood collection but no anesthesia. In preparation for the punction, either the animal or its tail has to be exposed to a heat source (exposure to a lamp or dipping the tail in moderately warm 37°C water for 5–8 min), which leads to a (local) vasodilatation and a facilitated blood sampling (DIEHL et al., 2001).

This technique is not suitable for guinea pigs and hamsters because they have no tails.

Blood sampling by incision of the lateral tail vein of mice requires the same kind of preparation in order to induce local vasodilatation. Unlike the punction of the lateral tail vein, the vessel is cut superficially with a sharp razor blade. This method is described as easier to perform than the punction of this vein, but it may have the disadvantage of contamination by tissue fluid (U.S. Department of Health and Human Services – National Institute of Health, 2005).
Repeated blood samplings are possible if further incisions are made near the previous incision (DÜRSCHLAG et al., 1996).
This method is recommended by the Swiss regulations for samplings yielding up to 0.1mL of blood (Bundesamt für Veterinärwesen, BVET – Richtlinie 3.02).
This technique is not suitable for guinea pigs and hamsters because they have no tails.

Blood collection using punction of the *V.saphena* in mice and hamsters requires no general anesthesia, only effective immobilization, e.g. in a restraining tube. The *V.saphena* runs dorsally and then laterally over the tarsal joint. Shaving the skin in this area and disinfection with 70% ethanol make the vessel clearly visible. The hind leg is immobilized in an extended position by applying gentle downward pressure immediately above the knee joint. A 25-gauge (0.6 mm) needle is used for the punction. A drop of blood forms immediately at the puncture site. Blood can be collected with a microcapillary tube for small samples (50-100µL) or a Microvette® collection tube (Sarstedt, D-51588 Nümbrecht, Germany) for larger samples. The bleeding can be stopped by applying gentle pressure to the puncture site or releasing the grip on the hind leg.
Furthermore, the removal of the clot allows the repeated collection of small amounts of blood (HEM et al., 1998).
If this method is carried out to collect samples larger than 100µL, it requires a more experienced technician compared to the blood sampling from the tail vein or the retrobulbar venous plexus of the eye (U.S. Department of Health and Human Services – National Institute of Health, 2005).
Punction of the lateral saphenous vein in guinea pigs permits the repeated blood samplings that are necessary in, e.g., pharmacokinetic studies. The guinea pig has to be restrained by a second person who extends the hind leg. Gentle pressure above the punction area leads to congestion of the saphenous vein (DIEHL et al., 2001).
The ideal punction area is found at the caudal side of the hind leg in the region between the *spatium inguinale* and the tarsal joint directly above the confluence of the *ramus cranialis* and the *ramus caudalis* of the vein. The needle has to be inserted at an angle of 30° to the crural axis and pushed forward from the ventral to the dorsal direction (HEIN, 2001).
Some authors recommend that blood be collected from the medial saphenous vein using a 25-gauge hypodermic needle attached to a 3mL syringe between the ankle and the knee joint (CARRAWAY and GRAY, 1989).
This technique permits blood collection without using anesthesia. Repeated blood samplings are also possible. Nevertheless, some authors call this method of collecting blood from guinea pigs a difficult procedure (HARTMANN et al., 1995).

Punction of the lateral tarsal vein can be used in hamsters held in tight restraint. To immobilize the hamster, the loose skin of the neck over the animal's shoulder is grasped. It is essential to cause congestion of the vein by exerting pressure on the extended hind leg. The

25-gauge (or smaller) hypodermic needle is inserted into the vein and blood can be collected into a blood collection tube (FIELD and SIBOLD, 1999).

The required preparation and restraint of mice for blood collection from the foot vein is analogous to the saphenous vein technique. The hind leg is extended, using the middle finger and forefinger. The dorsum of the foot is rubbed gently in order to locate a clearly visible vein and facilitate blood flow. Shaving of the skin is not necessary. The punction is carried out with a 25-gauge hypodermic needle, and 40µL of blood can be collected with a Microvette® blood collection tube (DOEING et al., 2003).
Other authors do not recommend this technique because of the sensitivity of the foot pad region and the risk of infection due to bedding contaminated by urine or feces (First report of the BVA/FRAME/RSPCA/UFAW/ joint working group on refinement, 1993).

Punction of peripheral ear vessels is recommended by Swiss legislation as a blood collection technique for guinea pigs. This method is suitable for repeated blood collections and yields blood amounts averaged out at 100 to 350 µL (Bundesamt für Veterinärwesen, BVET – Richtlinie 3.02).
Some authors recommend the application of local anesthetic cream 20–30 min before the punction takes place. This prevents the animal from shaking its head during the blood collection and eases the procedure (DIEHL et al., 2001).
The limited amount of blood that can be collected using this method can be viewed as a disadvantage, even though the method is repeatable. This method cannot be recommended if large volumes are required.

Incision of the marginal ear vein is also permitted as a method for collecting blood from guinea pigs, according to BVET Guideline 3.02. A transversal cut is made through the vein (Bundesamt für Veterinärwesen, BVET – Richtlinie 3.02).
This method enables the collection of a moderate volume of blood but is only permitted for a single blood sampling per vein. For this reason, the technique appears to be unfavorable or unsuitable if large volumes or repeated collections are required.

Punction of the penile vein is carried out in anesthetized guinea pigs. The penis of the unconscious animal is excavated and the dorsal vein of the penis is punctured.
No specific information can be given about this technique, because no papers describing this method were found.
This technique is not recommended because of its potential for adverse effects, e.g. temporary blockage of the urethra caused by thrombosis (First report of the BVA/FRAME/RSPCA/UFAW/ joint working group on refinement, 1993).

These non-terminal techniques are suitable for repeated blood samplings with the exception of the technique of the punction of the penile vein, which cannot be recommended.
Most of these methods (except for amputation of the tail tip) have the advantage that no anesthesia is required. The possibility of contamination by tissue fluid in the cases of tail tip amputation and lateral tail vein incision may also be noted as a disadvantage.
In addition, these methods do not permit blood samplings of large amounts (>200µL), a fact that restricts their use in certain cases.

If **moderate to large blood volumes** are required, the punction of the *V.jugularis*, the *V.facialis* or the retrobulbar venous plexus are the non-terminal techniques that are available today. A description of the punction of the retrobulbar venous plexus will not be given again here, because this method has already been described in the introductory section on the mouse.
No descriptions of the punction of the sublingual vein in mice could be found in the scientific literature. Therefore this technique is not mentioned here.

A successful punction of the jugular vein calls for an experienced technician.
The anesthesia of the mouse is essential. The unconscious animal is brought into a supine position and the head is dorsally hyperextended. The hyperextension is achieved by grasping the neck skin and pulling across the back of the technician's hand a string that is looped around the animal's upper incisors. This procedure leads to the congestion of the head and cervical veins. The jugular vein of the mouse is located 2–4mm laterally to the sternoclavicular junction. Shaving the neck area is useful because it makes the vein clearly visible. The skin is disinfected using 70% ethanol.
A 25-gauge hypodermic needle with a 1mL syringe is inserted 1-3mm deep and 2–4mm laterally to the sternoclavicular junction. The aspiration during the withdrawal should be carried out slowly in order to avoid collapse of the relatively small vessel (HOFF, 2000).
This technique must be carried out by a skilled person who is well trained. Otherwise the procedure will not be successful. The requirement for a high level of skill can be considered a disadvantage of this technique.
Guinea pigs were brought in a supine position and the hind limbs and the left forelimb were immobilized. The head is controlled by exerting pressure on the rami of the mandible by the thumb of the operator. The puncture site is found in the hollow of the right shoulder above the collarbone. The skin is disinfected using alcohol. A 24 gauge 5/8-in needle with 1-mL syringe is inserted in the puncture site and negative pressure is applied by drawing back the plunger of the syringe slightly. After blood collection is completed the apply of pressure on the puncture site is recommended to avoid haematomas. Adequate restraint is also essential. Up to 2.5mL of blood are collectable using this technique (SHOMER et al., 1999).
The implantation of a jugular catheter in guinea pigs and hamsters allows repeated blood collections at short intervals but requires a relatively high expenditure of time and material. The implantation has to be carried out under general anesthesia. Furthermore, the administration of heparin is necessary in order to keep the catheter permanently permeable (Bundesamt für Veterinärwesen, BVET – Richtlinie 3.02). This may influence specific blood parameters as well as the study results.
This technique is infrequently used even though it is suitable for repeated blood samplings of various volumes (TERRIL and CLEMONS, 1998).
If single samplings or only a few repeated collections are required, other techniques seem to be easier and less time-consuming.

A recently developed method is the punction of the *V.facialis* (also called mandibular or submandibular punction). The vein is situated near the *angulus mandibularis,* where a small circular, almost hairless point with a tactile hair is visible. The vein is located beneath this point and can be punctured here. The *V.facialis* and the *V.maxillaris* lead into the jugular vein. Both veins and also the *V.temporalis superficialis* (which leads into the maxillary vein) are identified by some authors as suitable locations for punction (video by Medipoint Inc., 2005).

The animal is firmly grasped by the scruff of the neck skin, which leads to partial stasis of the head veins, while the tail is held between the little finger and the ring finger. The head and the body of the animal have to be aligned along a straight line.
The area of the jaw slightly behind the almost hairless circular point is the right location for punction. After the blood sampling is completed, the grasp of the neck skin is released and the bleeding stops. This technique enables the collection of volumes of 100–200µL within 15–20 seconds from a mouse that is not anesthetized (Strebel, 2005), personnel communication).
GOLDE et al. report that a volume of 200-500µL per mouse can be collected (GOLDE et al., 2005).
The volume of blood collected can be adjusted by changing the size of the cannula used. To avoid collecting overly large volumes, the utilization of a 20-gauge or smaller needle is recommended (U.S. Department of Health and Human Services, National Institute of Health, 2005).
GOLDE et al. recommend the use of 4mm or 5mm standard fingertip lancets to facilitate the control of the depth of punction (GOLDE et al., 2005).
Even though the procedure is relatively quick and associated with a low degree of material investment, the possibility of mixing venous and arterial blood may be a disadvantage (U.S. Department of Health and Human Services; National Institute of Health, 2005).
This technique might be regarded critically because of the mouse anatomy. As shown in an illustration of POPESKO et al. (illustrated in the Annex, figures 17 and 18), wide areas of the *VV.facialis*, *temporalis superficialis*, *maxillaris* and *jugularis externa* are covered by relatively large glandular structures (*glandula lacrimalis externa orbitalis*, *glandula parotis* and *glandula mandibularis*) that are damaged during the blood collection procedure (POPESKO et al., 1992). As a result, saliva or tissue fluid probably contaminate the blood sampling and have a negative influence on its quality. In addition, other important structures, especially the *lymphonodi mandibulares* as well as different branches of the facial nerve, may be damaged by punction. This potential damage of important structures may influence the animal's well-being or may cause (severe) pain. The punction is carried out in a non-visible vein, which means it is impossible to avoid damaging non-venous structures.
During the blood collection, the blood moistens the fur of the animal, which may contaminate the sample. Shaving of the skin could avoid this possibility, but shaving in the head region without anesthesia or sedation leads to an increase of stress in the mouse.
Furthermore, anesthesia should be recommended if large samples are collected, in order to reduce distress of the animal.
Because of this method's current state of development, only a few studies report the use of this technique. Therefore its advantages or disadvantages (e.g. the degree of tissue damage, influence on different measurements etc.) cannot yet be clearly assessed.
A summary of all of the available blood sampling techniques for mice, guinea pigs and hamsters that have been described above is presented in Tables 7, 8 and 9 of the Annex.

11.2 Summary table of described blood collection techniques per species

The punction of the sublingual vein in mice and hamsters is included in this table.

Table 7: Summary of described blood collection techniques in mice

Technique	Terminal	Anesthesia required	Collectable volume	Max. volume / Reference[3]	Repeatable procedure[2]	remarks difficulties	recommended in BVET-RL 3.02
cardiac puncture	yes	yes	+++	9] 0.7–1mL 48] 0.3mL	–		no
decapitation	yes	no	+++	n.i.	–	contamination with tissue fluid or saliva possible	yes
punction of *V.cava caudalis*	yes	yes	+++	1] 1.6-2.5mL	–	time-consuming	no
punction of aorta	yes	yes	+++	28] 3% of body weight	–	time-consuming	no
punction of *V.axillaris*	yes	yes	+++	24] 1–2mL 49] 1–1.5mL	–	time-consuming	
tail tip amputation	no	no	+	5] 0.1mL 8] 0.1–0.2mL 48] 0.3mL	–	removal of the clot for subsequent blood collections	yes
punction of lateral tail vein	no	no	+/++	8] 0.1–0.15mL 9] 0.04mL 48] 0.3mL	+	adequate restraint and heat source for (local) vasodilatation required	yes
incision of lateral tail vein	no	no	+	5] <0.1mL 10] 0.04–0.15mL 24] 0.5–1mL	+	contamination with tissue fluid possible	yes
punction of *V.saphena*	no	no	+/++	19] 0.05–0.1mL 9] 0.04mL	+	requires experience	no
punction of foot vein	no	no	+	9] 0.04mL	+	requires experience	no
punction of *V.jugularis*	no	no	++	48] 0.3mL	+	requires high level of experience	no
punction of *V.facialis*	no	(no)	++	43] 0.1–0.2mL 15] 0.2-0.5mL	+	contamination with tissue fluid and saliva; damage to glands and lymph nodes	no
punction of retrobulbar venous plexus	no	yes	+++	5] >0.1mL 24] 0.25mL 48] 0.3mL 14] 1ml	(+)	14d recovery/eye after punction; severe tissue damage possible; contamination with porphyrins	yes
punction of sublingual vein	no	yes	+++	0.3 mL	+	horizontal position during collection is essential	no

Legend: 1: + = minimal volume; ++ = moderate volume; +++ = large volume
2: + = repeatable; – = single collection; (+) = restriction
3: number of reference and maximum collectable volume are quoted
n.i.: no information given in references

Table 8: Summary of described blood collection techniques in guinea pigs

Technique	Terminal	Anesthesia required	Collectable volume	Max. volume / Reference[3]	Repeatable procedure[2]	remarks difficulties	recommended in BVET-RL 3.02
punction of peripheral *V.saphena*	no	no	+/++	18] 3mL 6] 3mL (*V.saphena med*.)	+	Requires experience	yes
punction of peripheral ear vessels	no	no	+/++	5] 0.1–0.35mL	+	application of local anesthetic cream prevents head-shaking	yes
incision of marginal ear vein	no	no	++	48] 5mL	−	requires experience	yes
punction of the heart	yes	yes	+++	48] 5mL	(+)	second collection has to be terminal procedure; requires experience	yes
punction of the jugular vein	no	no	+++	43] 2.5mL	+	adequate restraint and pressure to the puncture site (2min) to avoid haematoma	no
jugular or femoral catheter	no	yes	+++	46] 5mL	+	expenditure of time / material; heparin necessary for permeability	yes
retrobulbar punction	no	yes	+++	46] 5mL	(+)	14d recovery after punction; severe tissue damage possible	yes

Legend: 1: + = minimal volume; ++ = moderate volume; +++ = large volume
2: + = repeatable; − = single collection; (+) = restriction
3: number of reference and maximum collectable volume are quoted

Table 9: Summary of described blood collection techniques in hamsters

Technique	Terminal	Anesthesia required	Collectable volume	Max. volume / Reference[3]	Repeatable procedure[2]	remarks difficulties	recommended in BVET-RL 3.02
abdominal *V.cava*	yes	yes	+++	n.i.	−	time-consuming	no
aorta	yes	yes	+++	n.i.	−	time-consuming	no
punction of the heart	yes	yes	+++	46] 0.3mL	(+)	second collection has to be terminal procedure; requires experience	yes
punction of lateral tarsal vein	no	no	+	n.i.	+	restraint and experience required	no
tail tip amputation	no	no	+	5] 0.1mL	−	removal of the clot for subsequent blood collections	yes
punction of *V.saphena*	no	no	+/++	n.i.	+	requires experience	no
jugular catheter	no	yes (implantation)	+++	46] 0.3mL (punction)	+	expenditure of time / material; heparin necessary for permeability	yes
punction of the retrobulbar venous plexus	no	yes	+++	46] 0.3mL 36] 3mL	(+)	14d recovery after punction; severe tissue damage possible	yes
punction of sublingual vein	no	yes	+++	0.3 mL	+		no

Legend: 1: + = minimal volume; ++ = moderate volume; +++ = large volume
2: + = repeatable; − = single collection; (+) = restriction
3: number of reference and maximum collectable volume are quoted
n.i.: no information given in references

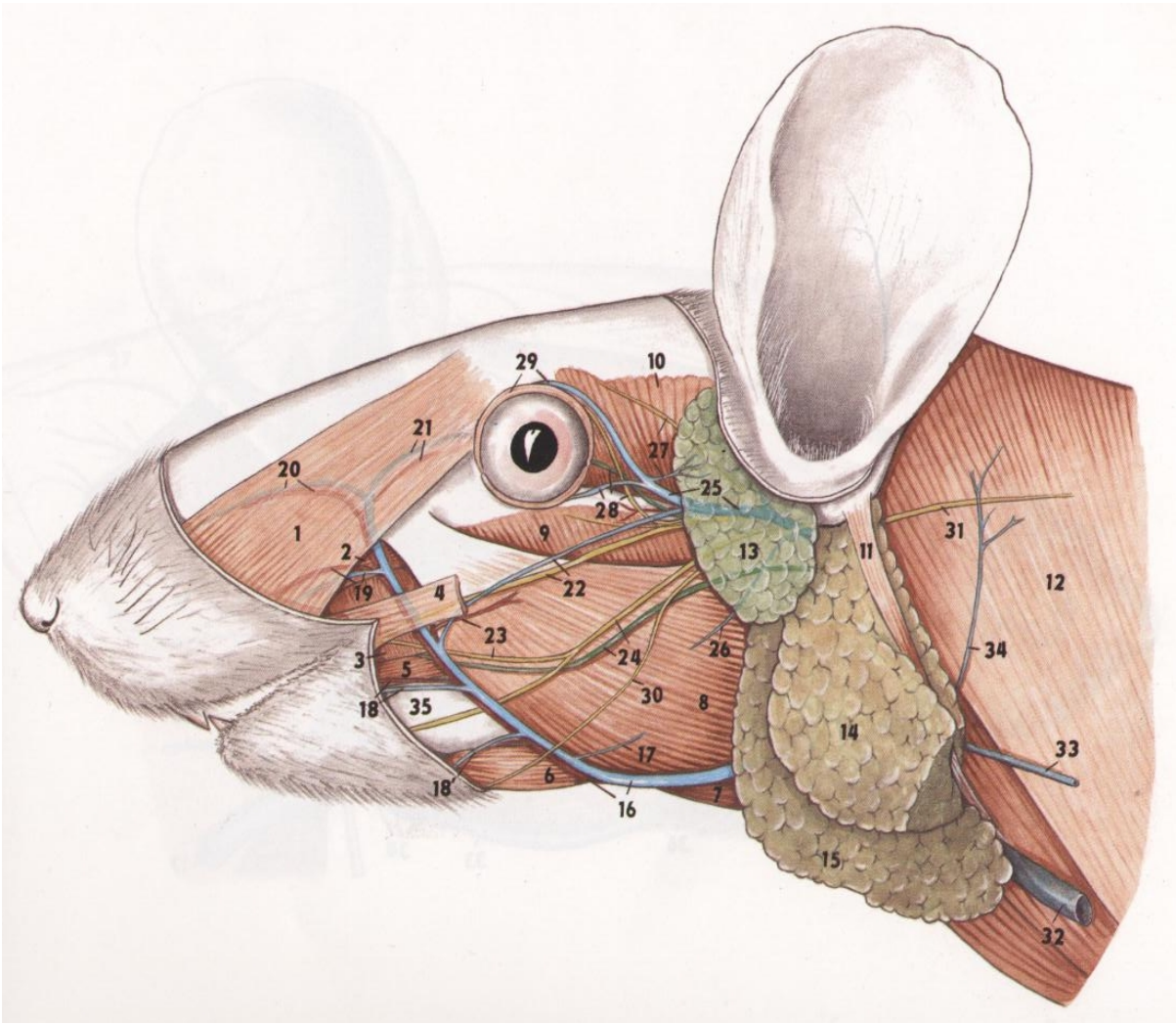
11.3 Summary of investigations of blood parameter differences

Table 10: Summary of investigations concerning the influence of technique on blood parameters

Authors	Species	Compared blood collection sources	Parameters with statistical significance	Remarks / recommendations
ANGELOW et al., 1984	rat	retrobulbar venous plexus vs. sublingual vein	(SL) ↑ = hematokrit, total protein (SL) ↓= triglycerides	Incision of sublingual vein
DAMERON et al., 1992	rat	retrobulbar venous plexus vs. *V.cava*	(RB) = prothrombin time and partial thromboplastin time prolonged	The authors do not recommend the retrobulbar blood collection technique if plasma coagulation times are measured
HERCK et al., 2001	rat	retrobulbar venous plexus vs. *V.saphena* vs. tail vein	(SA) ↑↑ = erythrocyte counts (RB) ↓↓ = hemoglobin, hematokrit (RB) ↑↑ = pCO^2, sodium (RB) ↓↓ = pH, potassium	Authors assume less erythrocyte damage and less influence on cellular barriers by retrobulbar punction
MAHL et al., 2000	rat	retrobulbar venous plexus vs. sublingual vein	(RB) ↓↓ = lymphocyte counts (RB) ↑↑ = neutrophil counts, creatin kinase, aspartate aminotransferase	Punction of sublingual vein (refined method) The authors assume that retrobulbar blood sampling causes more severe tissue damage than the sublingual method does
Doing et al., 2003	mouse	heart vs. tail vein vs. foot vein vs. V.saphena	(HE) ↑↑ = total leukocyte counts	Gender dimorphism: male mice showed lower level of neutrophil counts compared to female mice
SCHNELL et al., 2002	mouse	heart vs. V.cava vs. retrobulbar venous plexus	(RB) ↑↑ = erythrocyte counts, hemoglobin, hematokrit (HE) ↑↑ = total leukocyte counts, lymphocyte counts (VC) ↓↓ = albumin (RB) ↑↑ = creatin kinase (RB) ↑↑ = aldolase, transaminase, alkaline phosphatase (RB) ↑↑ = aspartate aminotransferase (VC) ↓↓= aspartate aminotransferase	Authors assume increased aspartate aminotransferase as a result of erythrocyte and tissue damage caused by retrobulbar punction; they recommend punction of the heart or V.cava as the ideal source

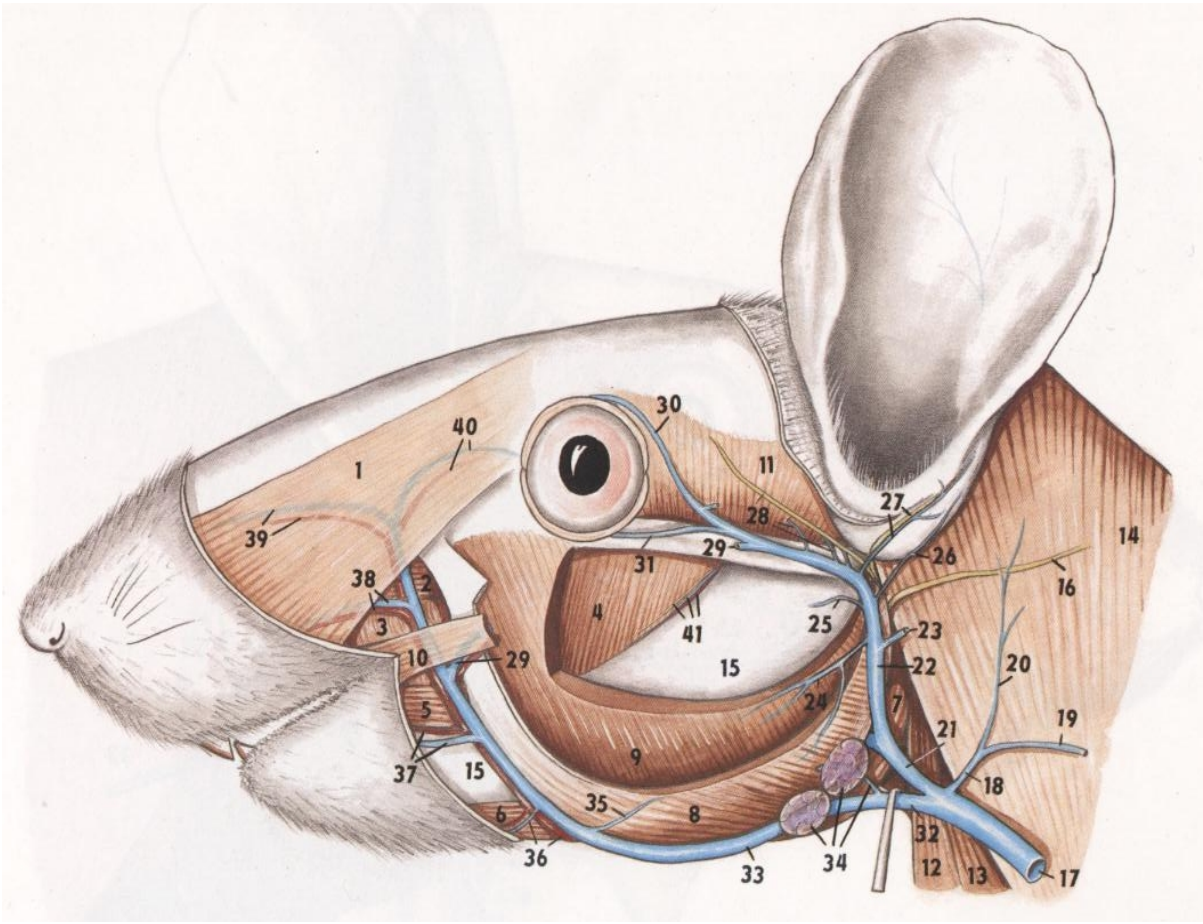
*Legend: ↑↑ = higher values; ↓↓= lower values; ─ = constant value; [↓], [↑] = statistically non-significant differences
RB = Retrobulbar venous plexus; SL = *V.sublingualis*; VC = *V.cava*; SA = *V.saphena*; HE = heart
vs. = versus

11.4 Anatomical illustration of lateral surface of the mouse



1 *m. levator nasolabialis* – nasolabial levator muscle
2 *m. levator labii superioris* – levator muscle of upper lip
3 *m. buccinator pars buccalis* – buccal part of buccinator muscle
4 *m. zygomaticus* – zygomatic muscle
5 *m. depressor labii inferioris* – depressor muscle of lower lip
6, 7 *m. digastricus* – digastric muscle
6 *venter rostralis* – rostral belly
7 *venter caudalis* – caudal belly
8, 9 *m. masseter* – masseter muscle
8 *pars superficialis* – superficial part
9 *pars profunda* – deep part
10 *m. temporalis* – temporal muscle
11 *m. parotidoauricularis* – parotidoauricular muscle
12 *m. trapezius pars cervicalis* – cervical part of trapezius muscle
13 *glandula lacrimalis extra orbitalis* – extra-orbital lacrimal gland
14 *glandula parotis* – parotid gland
15 *glandula mandibularis* – mandibular gland
16 *a. et v. facialis* – facial artery and vein
17 *v. massetericus* – masseteric vein
18 *a. et v. labialis inferior* – artery and vein of lower lip
18' *v. submentalis* – submental vein
19 *a. et v. labialis superior* – artery and vein of upper lip
20 *a. et v. dorsalis nasi* – dorsal nasal artery and vein
21 *a. et v. angularis oculi* – artery and vein of ocular angle
22 *v. transversa faciei, ramus buccalis dorsalis n. facialis* – transverse facial vein, dorsal buccal branch of facial nerve
23 *ramus anastomoticus inter nervus buccales, a. transversa faciei* – anastomosing branch between buccal nerves, facial transverse artery
24 *ductus parotideus, ramus buccalis ventralis n. facialis* – parotid duct, ventral buccal branch of facial nerve
25 *v. temporalis superficialis* – superficial temporal vein
26 *v. masseterica* – masseteric vein
27 *ramus glandularis, ramus auriculopalpebralis* – glandular branch, auriculopalpebral branch
28 *v. palpebralis inferior, ramus palpebralis, ductus glandulae lacrimalis extraorbitalis* – lower palpebral vein, palpebral branch, extraorbital glandular lacrimal duct
29 *v. supraorbitalis, m. orbicularis oculi* – supraorbital vein, orbicular ocular muscle
30 *ramus marginalis mandibulae n. facialis* – marginal mandibular branch of facial nerve
31 *ramus dorsalis n. accessorii* – dorsal branch of accessory nerve
32 *v. jugularis externa* – external jugular vein
33 *ramus prescapularis* – prescapular branch
34 *ramus ascendens* – ascending branch
35 *mandibula* – mandible

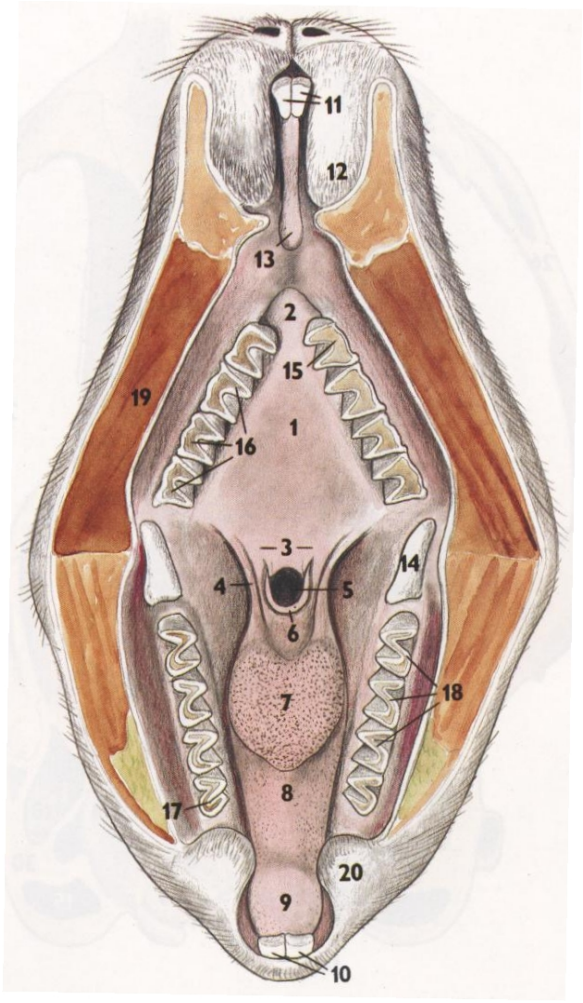
Figure 17: Lateral surface of mouse head, superficial layer (POPESKO et al., 1992)



1 *m. levator nasolabialis* – nasolabial levator muscle
2 *m. levator labii superioris* – levator muscle of upper lip
3, 4 *m. buccinator* – buccinator muscle
3 *pars buccalis* – buccal part
4 *pars molaris* – molar part
5 *m. depressor labii inferioris* – depressor muscle of lower lip
6, 7 *m. digastricus* – digastric muscle
6 *venter rostralis* – rostral belly
7 *venter caudalis* – caudal belly
8, 9 *m. masseter* – masseter muscle
8 *pars superficialis* – superficial part
9 *pars profunda* – deep part
10 *m. zygomaticus* – zygomatic muscle
11 *m. temporalis* – temporal muscle
12 *m. sternocephalicus pars mastoidea* – mastoid part of sternocephalic muscle
13 *m. cleidocephalicus pars mastoidea* – mastoid part of cleidocephalic muscle
14 *m. trapezius pars cervicalis* – cervical part of trapezius muscle
15 *mandibula* – mandible
16 *ramus dorsali s. accessorii* – dorsal branch of accessory nerve
17 *v. jugularis externa* – external jugular vein
18 *v. cervicalis superficialis* – superficial cervical vein
19 *ramus prescapularis* – prescapular branch
20 *ramus ascendens* – ascending branch
21 *v. maxillaris* – maxillary vein
22 *v. temporalis superficialis* – superficial temporal vein
23 *ramus parotideus* – parotid branch
24 *v. masseterica* – masseteric vein
25 *ramus massetericus* – masseteric branch
26 *v. auricularis caudalis* – caudal auricular vein
27 *v. auricularis rostralis, n. auricularis rostralis* – rostral auricular vein, rostral auricular nerve
28 *ramus auriculopalpebralis, rami temporales superficiales* – auriculopalpebral branch, superficial temporal branches
29 *v. transversa faciei* – transverse facial vein
30 *v. supraorbitalis* – supraorbital vein
31 *v. palpebralis inferior* – lower palpebral vein
32 *v. linguofacialis* – linguofacial vein
33 *v. facialis* – facial vein
34 *ramus glandularis, lnn. mandibulares* – glandular branch, mandibular lymph nodes
35 *ramus massetericus* – masseteric branch
36 *a. facialis, v. submentalis* – facial artery, submental vein
37 *a. et v. labialis inferior* – artery and vein of lower lip
38 *a. et v. labialis superior* – artery and vein of upper lip
39 *a. et v. dorsalis nasi* – dorsal nasal artery and vein
40 *a. et v. angularis oculi* – artery and vein of ocular angle
41 *a., v. et n. buccalis* – buccal artery, vein and nerve

Figure 18: Lateral surface of mouse head, deep layer (POPESKO et al., 1992)

11.5Illustration of oral cavity of a guinea pig



1 *palatum durum* – hard palate
2 *tuberculum medianum palati* – medial tubercle of palate
3 *palatum molle* – soft palate
4 *arcus palatoglossus* – palatoglossal arch
5 *ostium intrapharyngeum* – intrapharyngeal opening
6 *epiglottis* – epiglottis
7 *torus linguae (radix)* – torus of tongue (root)
8 *corpus linguae* – body of tongue
9 *apex linguae* – apex of tongue
10 *dentes incisivi inferiores* – lower incisors
11 *dentes incisivi superiores* – upper incisors
12 *panniculus intraoralis labii superioris* – intraoral panniculus of upper lip
13 *crista incisiva palati* – incisive crest of palate
14 *processus condylaris mandibulae* – condylar process of mandible
15 *dens premolaris (III) superior* – upper premolar tooth (third)
16 *dentes molares superiores* – upper molar teeth
17 *dens premolaris (III) inferior* – lower premolar tooth (third)
18 *dentes molares inferiores* – lower molar teeth
19 *m. masseter* – masseter muscle
20 *panniculus intraoralis labii inferioris* – intraoral panniculus of lower lip

Figure 19: Oral cavity of a guinea pig (POPESKO et al., 1992)

11.6 Individual hematology and clinical chemistry results of hamsters

Table 11: Hematology parameters of hamsters (sublingual method)*

	hamster 6	hamster 7	hamster 8	hamster 9	hamster 10	mean
WBC (x10^9 cells/L)	7.2	6.8	5.44	5.92	7.64	6.6
RBC (x10^{12} cells/L)	9.07	9.03	9.98	10.03	9.86	9.59
HGB (mmol/L)	10.2	10.2	11.3	11.5	11.1	10.86
HCT (mmol/L)	0.47	0.47	0.53	0.53	0.51	0.50
MCV (fL)	52.3	51.8	53.4	52.9	51.6	52.4
MCH (fmol)	1.12	1.13	1.14	1.14	1.12	1.13
MCHC (mmol/L)	21.45	21.74	21.27	21.65	21.75	21.57
Neutr. (x10^9 cells/L)	0.87	1.47	0.58	0.51	0.98	0.88
Neutr. (%)	12.1	21.6	10.6	8.6	12.8	13.14
Lymph. (x10^9 cells/L)	6	4.93	4.53	5.1	6.13	5.34
Lymph. (%)	83.4	72.5	83.3	86.1	80.2	81.1
Mono (x10^9 cells/L)	0.01	0.02	0.01	0.01	0.01	0.01
Mono (%)	0.1	0.3	0.2	0.1	0.1	0.16
Eos (x10^9 cells/L)	0.17	0.19	0.12	0.09	0.27	0.17
Eos (%)	2.4	2.7	2.3	1.5	3.5	2.48
Baso (x10^9 cells/L)	0.04	0.06	0.03	0.1	0.09	0.06
Baso (%)	0.6	0.8	0.5	1.7	1.2	0.96
PLT (x10^9 cells/L)	1238	1125	972	994	868	1039

Legend: * = automatically examined by ADVIA 120 (Bayer Diagnostics)

Table 12: Clinical chemistry parameters of hamsters (sublingual method)*

	hamster 1	hamster 2	hamster 3	hamster 4	hamster 5	mean
Na (mmol/l)	135.4	134.8	132.6	134.4	133.7	134.18
K (mmol/l)	5.68	6.17	5.88	5.16	6.57	5.89
CL (mmol/l)	96.2	92.2	94.4	92.2	93.9	93.78
Ca (mmol/l)	2.9	2.84	2.85	2.89	2.96	2.89
SGOT (IU/L)	43	37	55	37	62	46.8
SGPT (IU/L)	70	55	78	70	104	75.4
ALP (IU/L)	108.9	110.8	101.1	120.5	109.4	110.14
Crea (µmol/L)	34	39	39	34	34	36
Glu (mmol/L)	5.55	6.93	9.16	5.5	9.11	7.25
Urea (mmol/L)	6.23	7.14	6.48	6.97	8.03	6.97
Ttp (g/L)	62	65	66	68	65	65.2
Chol (mmol/L)	3.55	2.97	3.58	3.63	3.24	3.39
Tbil (µmol/L)	7.5	7	7.8	8.6	7.6	7.7
CK (IU/L)	743	514	684	293	249	496.6
PO4 (mmol/L)	1.58	1.65	1.51	1.56	1.96	1.65
Mg (mmol/L)	1.28	1.29	1.31	1.35	1.32	1.31
Alb (g/L)	40.5	40.9	41	42.7	42.1	41.44
TG5 (mmol/L)	2.4	2.58	2.26	2.85	2.85	2.59

Legend: * = automatically examined by SYNCHRON CX5 Analyzer

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13 Curriculum vitae

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